REMARKS AND ARGUMENTS:

Claims 6, 7 and 21 – 58 have been cancelled without prejudice or disclaimer. Applicants reserve the right to reassert any combination of claims 6, 7 and 21-58 during the prosecution of this application or any continuing applications that may be filed.

Claim 1 has been amended by clarifying that the flocculent and/or coagulant is other than and in addition to the polyarylamine. Support for this feature is found in the original specification at least at paragraph [00055].

Claim 10 has been amended by clarifying that the amine modifier is different than the arylamine. Support for this feature is found in the original specification at least at paragraph [00025].

Applicants have fully considered the Office Action dated October 19, 2006. In view of the above amendments and following remarks, Applicants respectfully request reconsideration of the application, withdrawal of the rejections, and issuance of a Notice of Allowance.

Election/Restriction comments:

The Office's comments regarding election and inventorship are noted.

Claim interpretation:

Applicants respectfully disagree with the Examiner's claim interpretation. According to the Examiner, paragraph [0019] of the instant specification defines the term arylamine as "any aromatic amine that is capable of reaction with an aldehyde to form a polymer." According to the Examiner, melamine compounds read on this definition since they are aromatic and capable of reaction with formaldehyde to form a polymer.

Applicants do not dispute the inclusion of this recitation within the specification. The claims, however, recite an arylamine as being selected from the group: "aniline, alkylanilines, phenylenediamines, aminophenols, methylenedianiline, homologues of methylenedianiline, and mixtures thereof." Those having ordinary skill in the art would recognize that melamine compounds are not members of this group. Accordingly, melamine compounds do not read on the polyarylamine polymers of the instant claims.

Rejection of claims 1-5 and 8-20 under 35 USC §112, second paragraph, as being indefinite.

It is respectfully requested that the rejection of claims 1 – 5 and 8 - 20 under 35 USC §112, second paragraph, as being indefinite be reconsidered for the reasons discussed below and be withdrawn. The Office argued that it is unclear whether the "flocculant and/or coagulant" is intended to modify or define the function of the polyarylamine polymer or is a further ingredient. The Office points to claim 20, where melamine formaldehyde is claimed as a flocculant.

The "flocculant and/or coagulant" of claim 1 is an element of the composition that is in addition to the polyarylamine. Claim 1 clearly recites a polyarylamine selected from a list of suitable polyarylamines and an other flocculant and/or coagulant. Support for this amendment may be found in paragraph [00055] of the application as originally filed. The plain language of the claim as amended, therefore, clearly states that the polyarylamine and the flocculant and/or coagulant are distinct. Accordingly, Applicants submit it is clear that the flocculant and/or coagulant is a further ingredient, because the claim states that flocculant and/or coagulant is "other" than the polyarylamine. Applicant agrees with the Examiner's statement that Claim 20 recites melamine formaldehyde as a flocculant. Claim 1, however, does not include melamine formaldehyde as a suitable polyarylamine component.

In claims 17, 18 and 19, the Office has argued that the ratio of the amine modifier:arylamine is unclear when the amine modifier and the arylamine are the same compound. Claim 10 stands as amended to recite, "...wherein the amine modifier is different from the arylamine." Accordingly, the ratios of Claims 17, 18, and 19 are clear because the amine modifier and the arylamine cannot overlap according to the claims.

In claim 5, the Office has argued that it is unclear how a hexamethylenetetramine is an aldehyde releasing agent.

As to the question by the Office as to whether hexamethylenetetramine is an aldehyde-releasing agent, the Applicant wishes to point out the statement, found in McDonnell *et al.*, Clin Microbiol Rev., 12(1):147 – 179 (1999), to the effect that:

> " Formaldehyde-releasing agents. Several formaldehyde-releasing agents have been used in the treatment of peritonitis (226, 273). They include noxythiolin

(oxymethylenethiourea), tauroline (a condensate of two molecules of the aminosulphonic acid taurine with three molecules of formaldehyde), hexamine (hexamethylenetetramine, methenamine), the resins melamine and urea formaldehydes, and imidazolone derivatives such as dantoin." (underlining added for emphasis)

The citations 226 and 273 are given as: (226) Hugo, W. B., and A. D. Russell. Types of antimicrobial agents. In A. D. Russell, W. B. Hugo, and G. A. J. Ayliffe (ed.), Principles and practice of disinfection, preservation and sterilization, 3rd ed., in press. Blackwell Science, Oxford, England, and (273) Lambert P A, Hammond S M. Potassium fluxes. First indications of membrane damage in microorganisms. Biochem Biophys Res Commun. 1973;54:796–799. Other references that show the release of formaldehyde from the reaction of hexamethylenetetramine and an acid are found in Synthesis, p. 161 (1979), and J. Organic Chemistry, 44:1678 (1979). Copies of the relevant references were included with the paper filed on December 19, 2006, and additional copies are enclosed herewith.

It is maintained, therefore, that hexamethylenetetramine was known in the art as an aldehyde-releasing agent at the time of the present invention.

Applicants respectfully request, in view of the above amendments and remarks, withdrawal of the 35 U.S.C. § 112, second paragraph rejections.

Rejection of claims 1 – 5, 8 – 12, and 14 – 20 under 35 USC §102(b) over U.S. Patent No. 4,422,944 to Selvarajan *et al.*

It is respectfully requested that the rejection of claims 1 – 5, 8 - 12 and 14 – 20 under 35 USC §102(b) as anticipated by U.S. Patent No. 4,422,944 to Selvarajan *et al.* be reconsidered for the reasons discussed below and be withdrawn.

To be anticipatory, a reference must disclose each element of the claimed invention. Present Claim 1 recites a polyarylamine polymer, wherein the arylamine is selected from the group consisting of anline, alkylanilines, phenylenediamines, aminophenols, methylenedianiline, homologues of methylenedianiline, and mixtures thereof. Additionally, Claim 1 recites an other flocculant and/or coagulant. Accordingly, Claim 1 requires both a polyarylamine and a distinct flocculant and/or coagulant.

With respect, the Applicant maintains that the polymer described by Selverajan *et al.* cannot fulfill both roles (that of flocculant and/or coagulant and polyarylamine polymers) because Claim 1 requires that the flocculant and/or coagulant is different than and in addition to the polyarylamine. The flocculant and/or coagulant must be different than the polyarylamine polymers. The Applicant maintains that the polymer described by Selverajan *et al.* cannot fulfill the role of both the flocculant and/or coagulant as well as the polyarylamine polymer according to the terms of the present claims. Consequently, the publication fails to teach all elements of the amended claims and that the rejection should be withdrawn.

Rejection of claims 1 – 5 and 8 – 20 under 35 USC §103(a) as obvious over Selvarajan *et al.*, in view of U.S. Patent No. 5,240,509 to Rey *et al.*

It is respectfully requested that the rejection of claims 1 – 5 and 8 – 20 under 35 USC §103(a) as obvious over Selvarajan *et al.*, in view of U.S. Patent No. 5,240,509 to Rey *et al.* be reconsidered for the reasons discussed below and withdrawn.

According to the Examiner, to the extent the Selvarajan polymers differ from the claims in the combination of the Selvarajan polymers with an additional flocculant and/or coagulant, the combination would have been obvious to one having ordinary skill in the art due to Rey's disclosure of treating water with compositions comprising a combination of melamine-formaldehyde and an inorganic flocculant or nonionic flocculant. Applicants respectfully traverse the rejection.

Amended Claim 1 recites the arylamine reactant as being selected from a list of specific arylamines formerly described in claim 7, now cancelled. Because neither Selvarajan nor Rey *et al.* teach or suggest the use of any arylamine other than melamine, which is not included in the useful arylamines of claim 1, they cannot teach the invention as claimed. Moreover, because the structure of melamine (a triamino triazine) is significantly different from the anilines, phenylenediamines, and amino phenols required in the present invention, one of ordinary skill in the art would have no reason to expect that the substitution of one of those compounds for the melamine required in Rey *et al.* would result in a successful treating agent for contaminated spray booth waters. Without a suggestion to make this modification of the Rey *et al.*

melamine polymer, the Rey *et al.* reference cannot be argued to make the present claims obvious.

Additionally, neither Selvarajan nor Rey teach the use of a flocculant and/or coagulant distinct from the polyarylamine polymer including arylamines selected from the list of Claim 1. There is no teaching, suggestion, or motivation provided by either reference that would lead one having ordinary skill in the art to combine the cited references and then add additional arylamine polymers to detackify paint in an aqueous system. Absent such motivation, the claims cannot be said to be obvious in view of the cited combination.

Accordingly, it is respectfully requested that the present ground of rejection be reconsidered and be withdrawn.

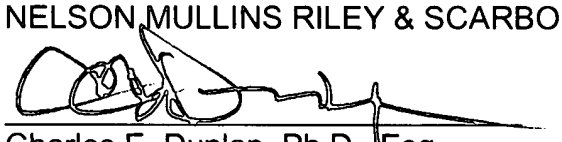
Request for reconsideration:

Applicants respectfully submit the application is in condition for allowance in view of the above amendments and remarks. If one or all of the claims are deemed to not be allowable, the Examiner is invited to call the undersigned attorney at the number given below for resolution of any remaining issues.

It is believed that no fees are due in conjunction with the filing of this response. If, however, it is determined that fees are due, authorization is hereby given to deduct those fees from Deposit Account No. 50-2548.

Respectfully requested,

NELSON MULLINS RILEY & SCARBOROUGH, LLP



February 19, 2007
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POTASSIUM FLUXES. FIRST INDICATIONS OF MEMBRANE DAMAGE IN MICRO-ORGANISMS.

P.A. Lambert, and S.M. Hammond

School of Biological Sciences, Thames Polytechnic, London, SE18 6PF, U.K.

Received August 2, 1973

SUMMARY

A method is described whereby the leakage of potassium ions can be measured from microbial cells which have been treated with membrane active antimicrobial compounds. The method involves the use of specific ion electrodes and does not require the cells to be removed from suspension. The effect of two membrane-active antimicrobials; cetrimide upon E.coli and amphotericin upon C.albicans is related to the leakage of phosphate and 260 nm material.

Membrane active antimicrobial agents have been shown to release various cytoplasmic constituents from treated cells. (1) Components which have been detected include 260 nm. absorbing material, by spectrophotometry, phosphate and pentoses, by colorimetric methods; and potassium ions by flame photometry. All these assay procedures require the removal of the treated cells from the suspension before an analysis can be made upon the supernatant solutions. We would like to report methods whereby the leakage of potassium from treated cells can be measured in situ using specific ion electrodes.

MATERIALS AND METHODS

Two types of potassium sensitive specific ion electrodes are commercially available; glass electrodes (2) and liquid membrane electrodes. (3) Glass electrodes must be used under conditions of constant pH, preferably pH 7 or above, and in the absence of sodium and ammonium ions. Liquid membrane electrodes may be used at any pH and, being far more selective than glass electrodes, may be used in the presence of sodium or ammonium ions, but have recently been shown to be affected by certain compounds. (4)

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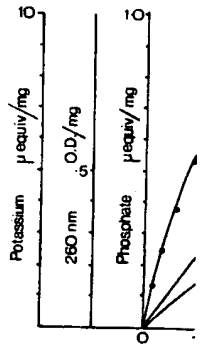

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We have used both electrodes to measure the potassium effluxes caused by the action of various antimicrobial agents upon bacterial and fungal suspensions.

Cells were harvested, washed twice in distilled water, and resuspended in appropriate buffer solution to give cell densities of 1mg./ml. dry weight. 20ml of the suspension was placed in a thermostatically controlled vessel, magnetically stirred, and the electrode inserted. The potential derived by the electrode was measured using a Vibret 46A mv/pH meter. The antimicrobial agent was added to the suspension and the potassium concentration was measured in situ by the electrode, readings being taken at ten second intervals.

RESULTS AND DISCUSSION

Figure 1 shows the leakage of potassium, phosphate and 260 nm.

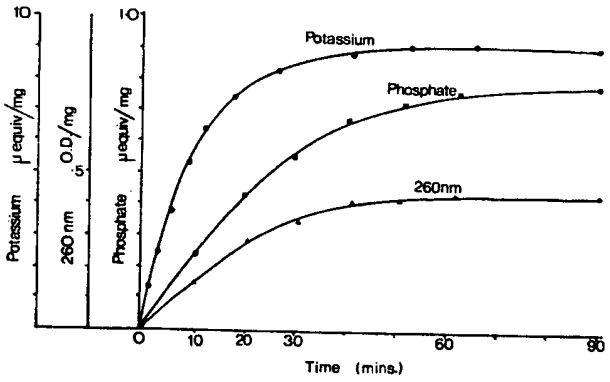

FIG. 1. Time course for leakage of components from E.coli. treated with 0.2mM. cetrimide at 25°C. & pH 7.

absorbing material caused by the action of 0.2 mM. cetrimide upon a suspension of E.coli. NCIB 8277 (10^9 cells/ml.) in TRIS/HCl buffer pH 7 at 25°C. 260 nm. material and phosphate were measured in the supernatant after removing the cells by centrifugation at 10,000 g. for 5 mins, 260 nm. absorbing material directly, while phosphate was determined by the method of King. (5) The results show that the initial effect of cetrimide upon E.coli under these conditions was to initiate a flux of potassium ions which was complete within

30 minutes. This was followed by a slower release of phosphate and 260 nm. material as reported by Salton (6) and Newton (7).

Figure 2 shows the effect of amphotericin B, a membrane active

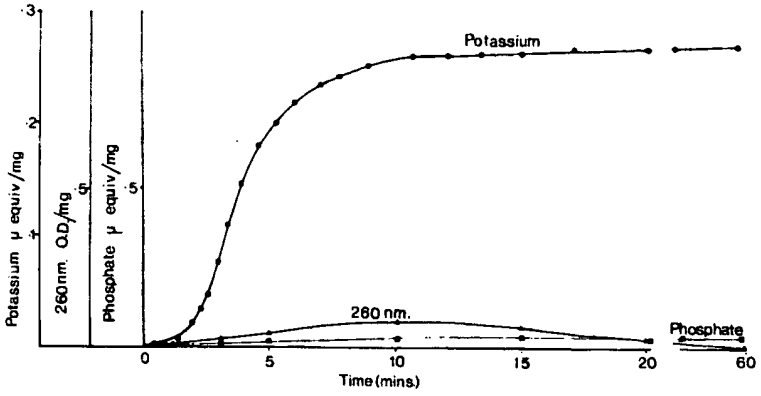

FIG.2. Time course for leakage of components from C.albicans treated with 20 μg amphotericin B at 25°C. & pH 6.

polyene antibiotic, against the yeast Candida albicans NTCC 713, detected by a Philips 560-K valinomycin-based potassium sensitive liquid membrane electrode. 20 μg of amphotericin B causes insignificant leakage of phosphate and 260 nm. absorbing material from a washed suspension of the yeast, (3×10^6 cells/ml.) but potassium leakage is marked.

Specific ion electrodes permit rapid accurate measurement of potassium leakage following treatment of washed microbial suspensions with membrane active antimicrobial agents. The leakage of potassium ions occurs extremely rapidly after treatment. We have detected leakage within 5 seconds using these methods. It is proposed that the efflux of this ion is one of the first indications of the changes induced in the selective permeability of microbial membranes by membrane active antimicrobial agents.

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6. Salton M.R.J. (1
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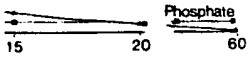

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Structure and Chemistry of the Aldehyde Ammonias. 3. Formaldehyde–Ammonia Reaction. 1,3,5-Hexahydrotriazine[1]

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Chemistry Division, Research Department, Code 385, Naval Weapons Center, China Lake, California 93555

Received December 12, 1978

The formaldehyde–ammonia reaction has been examined in D_2O solvent with the aid of ^1H and ^{13}C NMR spectroscopy. Reaction intermediates, including 1,3,5-hexahydrotriazine (2) and 1,3,5,7-tetraazabicyclo[3.3.1]nonane (6), are observed, and the mechanisms of their formation and conversion into hexamine are discussed. Solutions previously reported to contain 2 alone have been shown to be complex mixtures.

The formaldehyde–ammonia reaction under appropriate conditions leads quantitatively to hexamine (1, eq 1).[3] Despite

$$6CH_2O + 4NH_3 \longrightarrow \text{1} + 6H_2O \qquad (1)$$

its importance, it has received limited detailed study; understanding of this complex reaction sequence is tentative. In the present work ^1H and ^{13}C NMR spectroscopy have been employed to follow the reaction and identify some intermediates.

The most extensive recent study of the formaldehyde–ammonia reaction is that of Richmond, Myers, and Wright.[4] From chemical evidence they concluded that 1,3,5-hexahydrotriazine (2) is a reaction intermediate. This result is in

2

3, R = alkyl
3a, R = CH_3

agreement with the early reports of Duden and Scharff,[5] who first correctly assigned structure 1 to hexamine. The present study provides NMR evidence for formation of 2. Our previous studies on reaction of *n*-alkanals with ammonia showed that the ultimate reaction products are usually 2,4,6-trialkyl-1,3,5-hexahydrotriazines (3).[1,6] The kinetics of the formaldehyde–ammonia reaction have been examined by several workers.[7]

Results and Discussion

^1H and ^{13}C NMR Spectra of Formaldehyde–Ammonia Solutions. In the present work ^1H and ^{13}C NMR spectra of various D_2O solutions of ammonium-d_4 hydroxide and formaldehyde at 25 °C were determined at intervals. The ^1H spectra clearly reveal the rapid formation of hexamine and the presence of reaction intermediates (Figure 1). The methylene signals are grouped in two principal regions. Rather broad signals near δ 4.5 are attributed to NCH_2O-type methylenes; compare these signals with those of 1,3-perhydrotriazine (4) in which the C-2 methylenes appear at δ 4.55[8a] (Table I). Sharper, more defined signals appear near δ 3.5–4.0 and represent NCH_2N-type methylenes (with the exception of hexamine itself which appears as a singlet at δ 4.75).[9] Altering the formaldehyde–ammonia molar ratio from 1:4 to 3:2 provides the same products in each experiment, and their relative distribution at a given time is changed but slightly.

4 5

1,3,5-Hexahydrotriazine (2) exhibits a sharp singlet at 3.95. This value may be compared with the C-2 methylen signal of 1,3-hexahydrodiazine (5, δ 3.73)[8b] and the rin methine signal of 2,4,6-trimethyl-1,3,5-hexahydrotriazine [3a δ 3.80 (q)] in D_2O solvent (Table I). Hexahydrotriazine form rapidly and ultimately is the principal species present othe than hexamine. Initially, its concentration is much greate than that of hexamine, a fact supported by low temperatur (−10 °C) ^{13}C NMR spectra observations; e.g., the peak height ratio of 2/1 equals 3 after 1 h.

Signals attributed to 1,3,5,7-tetraazabicyclo[3.3.1]nonane (6) persist in the ^1H NMR spectra of formaldehyde–ammonia

6 7

solutions (Figure 1; other spectra obtained show this more clearly). The concentration of 6 is relatively much lower than that of 2 except in the earlier reaction stages where it appears to be nearly equal. The signals attributed to 6 appear as two single lines: one a collapsed AB quartet of the 2,4,6,8 ring methylene protons centered near δ 3.86; the second, a singlet of one-fourth relative intensity, is the bridge methylene at δ 3.81. The constant 4:1 ratio of these peaks is retained as their intensities vary and supports the structure assignment. The ^1H NMR spectra of numerous model compounds are similar to that of 6. For example, 3,7-dinitro-1,3,5,7-tetraazabicyclo[3.3.1]nonane (7) exhibits a bridgehead methylene singlet at δ 4.14; the ring methylenes appear as an AB quartet (Table I).[10]

The conformation of 6 is shown as a flattened chair–chair, which is favored in various heterocyclic bicyclo[3.3.1]nonanes.[10,11] To explain the observed collapsed AB quartet of 6, the ring axial and equatorial protons are seen as similarly deshielded since both are equidistant from the adjacent nitrogen p lobes, assuming the N-3 and N-7 lobes to be exocyclic to avoid their "rabbit ear" interaction. The N-3 and N-7 hydrogens thereby assume an axial configuration. Thus, the methylene hydrogens and nitrogen lone pair orbitals in 6 appear to be oriented like those in hexamine.

Broad signals near δ 4.5 are attributed, principally, to *N*-methylol-*O*-*d* derivatives. The breadth of these signals, which persists throughout the reaction, suggests a rapid steady-state turnover of these groups owing to their short half-lives. The intensity of these signals disappears more rapidly than that

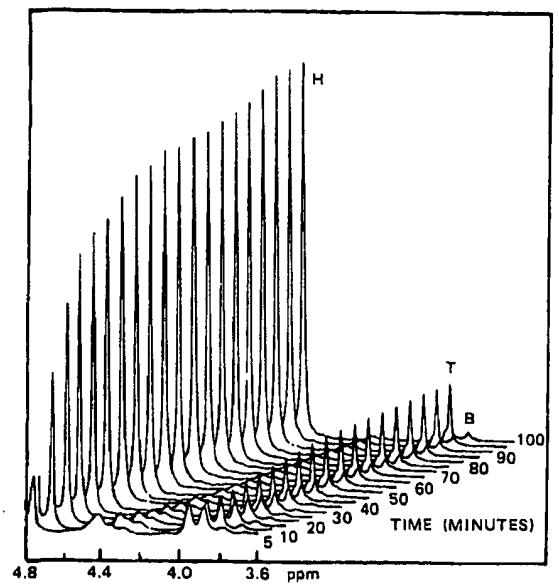


Figure 1. ^1H NMR spectra of D_2O solution of formaldehyde (1 M) and ammonium hydroxide (1 M) determined at various times after mixing (25 °C): H = hexamine (1); T = 1,3,5-hexahydrotriazine (2); B = 1,3,5,7-tetraazabicyclo[3.3.1]nonane (6).

Table I. ^1H and ^{13}C NMR Spectra in D_2O Solvent (25 °C)

compd no.	group	chemical shift, δ[a] ^1H	^{13}C
1	CH_2	4.75 (s)	74.5
2	CH_2	3.95 (s)	61.6
3a	CH	3.80 (q, J = 6.5 Hz)	67.3
4	C-2 CH_2	4.55 (s)[b]	
5	C-2 CH_2	3.73 (s)[c]	
6	C-2,4,6,8 CH_2	3.86 (q)[d]	66.6[e]
	C-10 CH_2	3.81 (s)	69.9[e]
7[f]	C-2,4,6,8 CH_2	4.96, 5.52 (q, J = 13 Hz)	68.5
	C-10 CH_2	4.14 (s)	64.9
8	C-2,2′,6,6′ CH_2	3.97 (s)[e]	67.0[e]
	C-4,4′ CH_2	3.95 (s)[e]	61.4[e]
	bridge CH_2		68.4[e]
9	C-2,4 CH_2		65.3[e]
	C-6,8 CH_2		66.3[e]
	C-10 CH_2		70.1[e]
	N-3 *exo*-CH_2		70.8[e]
12a	CH_2[g]	6.40 (s)	55.4
12b	CH_2[g]	5.77 (s), 6.38 (s), 7.03 (s)	46.2, 56.8, 65.4
13a[h]	C-2,8 CH_2	4.25, 5.81 (q)[g]	60.7[f]
	C-4,6 CH_2	5.30, 5.49 (q,[g] J = 13 Hz)	69.4[f]
	C-10 CH_2	4.59 (s)	68.1[f]
13b[h]	C-4,8 CH_2	4.33, 5.81 (q)[g]	59.9[f]
	C-2,6 CH_2	5.30, 5.53 (q,[g] J = 13 Hz)	70.1[f]
	C-10 CH_2	4.59 (s)	68.4[f]

[a] Sodium 3-(trimethylsilyl)propanoate internal reference for D_2O solutions, and tetramethylsilane for others. [b] Reported δ 4.40 in $CDCl_3$.[8] [c] Reported δ 3.63 in D_2O; reference compound was not stated.[9] [d] Center lines of collapsed AB quartet. [e] Suggested assignments. [f] $(CD_3)_2SO$ solvent; some data are also reported in ref 10a,b. [g] $(CD_3)_2CO$ solvent; some data are also reported in ref 10a,c. [h] Compound prepared by the procedure of Bachmann and Deno.[15]

of **2** and **6** (also observed in the ^{13}C NMR spectra).

The proton-decoupled Fourier transform ^{13}C NMR spectra of formaldehyde–ammonia solutions in D_2O provide data in agreement with those derived from proton spectra. Near the completion of the reaction, two principal signals persist, those

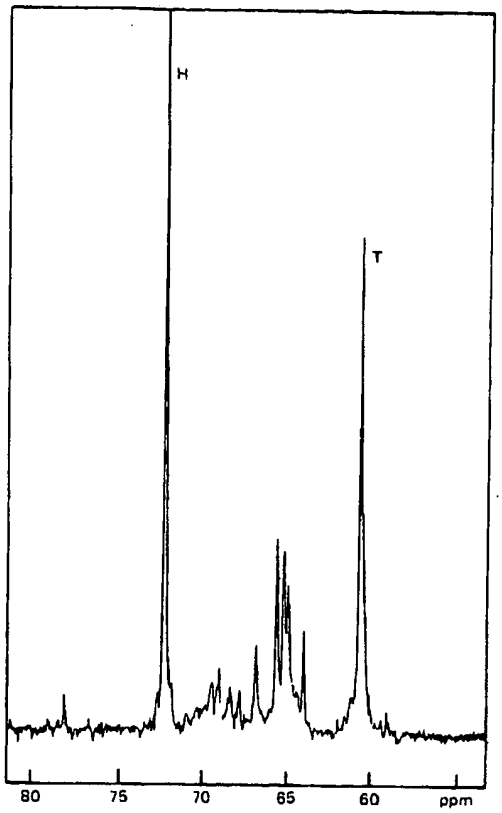
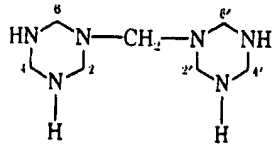


Figure 2. Proton-decoupled ^{13}C NMR spectrum of H_2O–D_2O solution of formaldehyde (5.7 M) and ammonium hydroxide (7.8 M) determined after 24 h elapsed reaction time at −10 °C: H = hexamine (1); T = 1,3,5-hexahydrotriazine (2).

of hexamine (**1**, δ 74.5) and 1,3,5-hexahydrotriazine (**2**, δ 61.6). Owing to the required signal-averaging time (~10–15 min), ^{13}C spectra obtained at early and intermediate reaction times are observed to be very complex with numerous lines to which it is difficult to match structure assignments. Spectra obtained at later reaction times are simpler and show the intense lines of **1** and **2** in addition to other weaker lines, most of which are grouped as a pattern of nine lines showing estimated relative intensity ratios of 4:4:2:2:2:1:1:1:1. These ratios are retained in three groups (4:1, 4:2:1, and 2:2:1:1) of diminished intensity as the reaction proceeds (Figure 2 shows a typical spectrum). One pair of lines (δ 66.6, 69.9; 4:1 ratio) is attributed to **6** (Table I). Stable intermediate compounds such as **8** (signal



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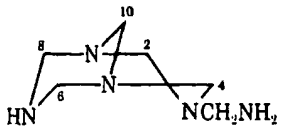

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intensity ratio 4:2:1) and **9** (ratio 2:2:1:1) would account for the remaining lines, although certain less plausible and less stable alternative structures could be written. A line at δ 61.4 (estimated relative intensity 2) seen as a shoulder on the triazine peak (Figure 2) suggests a structure very similar to **2**; the signal of the 4,4′ carbons of **8** would be expected to appear near δ 61.5. [A weak shoulder (δ 3.97) on the proton spectrum signal of **2** (Figure 1) could correspond to 2,2′,6,6′ ring methylene signals in **8**.] The spectrum of **9** should closely resemble that of **6** (suggested signal assignments are listed in Table I). The bis(nitramine) **7** has a spectrum similar to that of **6** (lines at δ 64.9 and 68.5 in $(CD_3)_2SO$ solvent[10b]), although nitro substitution affects the chemical shift values.

1,3,5-Hexahydrotriazine (2). The present study shows

1,3,5-hexahydrotriazine formation to occur in ammonia–formaldehyde reactions (Figures 1 and 2). At equilibrium it is the principal minor component in solution; hexamine is the major component. Our efforts to prepare pure 2, either neat or in solution, were unsuccessful. The material is converted into hexamine under a variety of conditions.

A substance known as **Henry solution**[12] is prepared by passing 1 mol equiv of ammonia gas into a solution of 40% formalin at 0 °C, followed by addition of sufficient solid potassium carbonate to cause separation of a floating oily layer. The oil is treated with additional potassium carbonate, decanted or centrifuged, and stored at low temperature.[4,12] Although the directions of Henry call for a 1:1 molar ratio of formaldehyde and ammonia, Wright[4] observed that this ratio could be varied without altering the chemical properties of the product.

Wright concluded from chemical evidence that Henry solution is largely aqueous 1,3,5-hexahydrotriazine (2, 50% by weight).[4] Henry believed his product to be trimethylolamine, $(HOCH_2)_3N$.[12] In the present study it is concluded that Henry solution contains some 2, in addition to other components, but rapidly changes to hexamine on aging. The ^1H and ^{13}C NMR spectra of Henry solution and the spectra of aqueous formaldehyde–ammonia solutions at comparable reaction points are virtually indistinguishable and are believed to contain the same components.

By examination of the proton NMR spectra of Henry solution prepared in D_2O and determined at intervals at 25 °C, the relative concentrations of the major components were determined. Figure 3 shows a plot of time vs. the approximate relative molar concentrations of 1, 2, and 6 (established by dividing the appropriate peak heights by the number of protons per signal; small amounts of 8 and 9 may be included in the concentration values for 2 and 6, respectively). It may be observed that after about 2 weeks the molar ratio of the three components is nearly equal and that the concentration of 2 remains high for about 1 month before the relative concentration of hexamine predominates significantly. The rate of reaction is accelerated greatly on warming. It is clear that Henry solution is a dynamic mixture. Wright's conclusion that it behaves as 1,3,5-hexahydrotriazine, although partiall correct, is applicable only to fresh solutions.

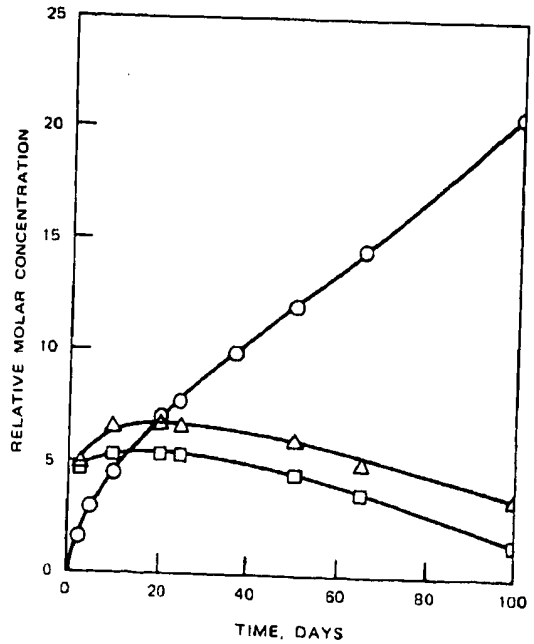


Figure 3. Plot of relative molar concentrations of Henry solution components hexamine (1) (○), 1,3,5-hexahydrotriazine (2) (△), and 1,3,5,7-tetraazabicyclo[3.3.1]nonane (6) (□) vs. time (25 °C). Data are calculated from ^1H NMR peak heights divided by the number of protons corresponding to the signal to obtain the concentration index.

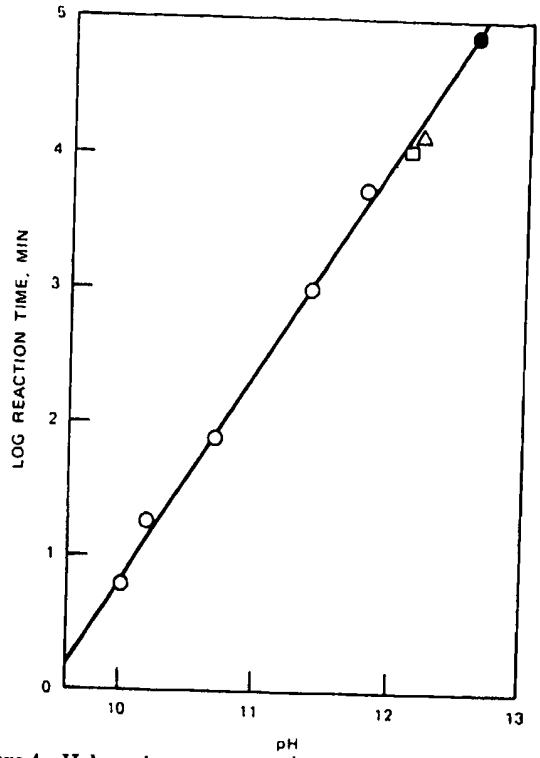


Figure 4. pH dependence of the logarithm of the hexamine formati rate (minutes elapsed to ^1H NMR peak height ratio of hexami (1)/1,3,5-hexahydrotriazine (2) = 4:1): (○) D_2O solutions of CH_2 ND_4OD only; (□) NaOH added; (△) Duden and Scharff solution; (Henry solution.

An important misconception regarding Henry solutio ("1,3,5-hexahydrotriazine solution") is the failure to recogniz its transitory shelf life. Thus, at present several chemica distributors market a product, prepared by Wright's proce dure,[4] labeled "hexahydro-*s*-triazine, 50% aqueous solution".[1] It is claimed to have a shelf life of 12–15 months when kep cool, or "several weeks" at ambient temperature.[4,13] Actually all samples of such materials which we have obtained smel strongly of ammonia and have been found to contain onl hexamine (~40% aqueous solution) and ammonium hydrox ide. Occasionally crystals of hexamine separate from these solutions. Presumably such solutions have aged or become warm excessively. Use of such samples in chemical reactions can lead to ambiguous results, as in the questionable report of a preparation of 1,3,5-hexahydrotriazinium nitrate.[14]

Henry solution has a much longer shelf life (as measured by its rate of hexamine formation from 2) than 1:1 formaldehyde–ammonia solutions; compare Figures 1 and 3. This is due to its high ammonia content and high pH (12.6). The effect of pH on a measure of the rate of hexamine formation is seen in Figure 4, which plots the log of time required for the molar ratio of hexamine to hexahydrotriazine to reach 2:1 vs. pH (D_2O solutions; data are in Table II). The pH remains reasonably constant at this stage of the reaction and may be measured quite accurately. The pH was varied by controlling the concentration of reactants or by addition of sodium hydroxide. Henry solution contains traces of potassium carbonate (0.2%), but this base at this low concentration has virtually no effect on the pH in comparison to the large amount of ammonia and amines present (Table II). Addition of formaldehyde or acid to Henry solution results in its rapid conversion to hexamine. Aqueous solutions of hexamine made acidic (pH 1) or basic (pH 12) revealed only the hexamine peak in NMR spectra after several hours. However, vigorous acid

Table II. Formaldehyde–Ammonia Reactions in D_2O at 25 °C: pH–Rate Profile Data

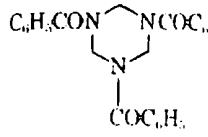
reactant concentration, M[a] $[CH_2O]$	$[NH_3]$	reaction time,[b] min	pH[c]
0.50	0.50	6	10.0
0.50	0.50[d]	10	10.1
0.67	0.67	18	10.2
0.50	1.00	90	10.7
4.7	4.7	1030	11.4
3.0	6.0	5700	11.8
2.8	2.8[e]	11 000	12.1
2.8	2.8[f]	13 400	12.2
15	15[g]	72 000	12.6

[a] Concentration of reactants in solution after initial mixing. [b] Time required for ratio of peak heights of ^1H NMR signals of hexamine and 1,3,5-hexahydrotriazine, respectively, to reach 4:1. [c] pH measured when peak height ratio of hexamine to 1,3,5-hexahydrotriazine signals = 4:1. [d] Solution 0.07 M in K_2CO_3. [e] Solution 0.28 M in NaOD. [f] Duden and Scharff solution. [g] Henry solution; concentrations of reactants calculated from data of ref 4; solution ~0.01 M in K_2CO_3.

treatment of hexamine (reflux several hours) results in complete degradation to formaldehyde and ammonia.[7a,g]

Attempts were made to isolate pure 1,3,5-hexahydrotriazine (2). Freshly prepared Henry solution was concentrated to dryness at near 0 °C to yield a white solid, aliquots of which were extracted with various cold solvents. When reconstituted in D_2O its ^1H NMR spectrum was very similar to that of the original, except for some slight decrease in the triazine peak intensity. Extraction with cold $CDCl_3$ produced similar results, except that the triazine peak was diminished considerably. Extractions into carbon tetrachloride or benzene-d_6 gave solutions showing the spectrum of hexamine only; 2 would be expected to be less soluble in these solvents. These preliminary results suggest that 2 exhibits thermal instability like that of 2,4,6-trialkyl-1,3,5-hexahydrotriazines (3),[1,6] most of which decompose rapidly at ambient temperatures. Based on observed relative NMR peak intensities, the bicyclotetramine 6 present in the reconstituted Henry solution appears to be relatively more stable than 2.

Duden and Scharff solution[5] is prepared by addition of 40% formalin to aqueous ammonium hydroxide prepared by addition of a molar excess of sodium hydroxide to aqueous ammonium chloride solution. These authors concluded that their solution contained 1,3,5-hexahydrotriazine since it reacted with benzoyl chloride to form the 1,3,5-tribenzoyl derivative 10, in addition to the acyclic product 11. These



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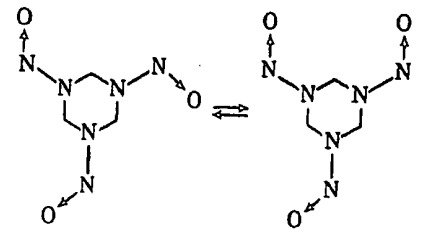
$C_6H_5CONHCH_2NHCH_2NHCOC_6H_5$

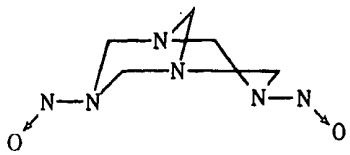
11

results were confirmed by Wright.[4] Duden and Scharff solution differs from Henry solution in that it is less concentrated in amine products and contains sodium chloride and sodium hydroxide. It forms hexamine more slowly than unmodified 1:1 formaldehyde–ammonia solutions of the same molarity and more rapidly than Henry solution. Wright attributed these rate differences to the greater amine (not ammonia) concentration in Henry solution.[4] We have now established that it is the presence of excess sodium hydroxide which accounts for the enhanced stability of Duden and Scharff solution since it raises the pH to a value (12.2) where hexamine formation is quite slow (Figure 4). Solutions of 1:1 formaldehyde–ammonia to which sodium hydroxide has been added to cause a pH increase also form hexamine very slowly (Figure 4). Added sodium chloride exhibits no significant salt effect on the observed hexamine formation rate, in agreement with the kinetic data of Ogata and Kawasaki.[7g,i]

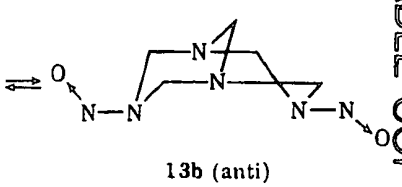
The chemical behavior of 1,3,5-hexahydrotriazine solutions (Henry and Duden and Scharff solutions) differs in some, but not all, respects from that of solutions of pure hexamine. The subject has been reviewed and studied by Wright.[4] For example, the preparation of 1,3,5-tribenzoyl-1,3,5-hexahydrotriazine (10) from fresh Henry solution contains little 1,3,5-tribenzoyl-1,3,5-triazapentane (11), whereas the preparation of 10 from hexamine contains larger amounts of the latter substance. Hexamine solution reacts with diazotized *m*-nitroaniline to yield 3,7-bis(3-nitrophenyldiazo)-1,3,5,7-tetraazabicyclo[3.3.1]nonane, whereas freshly prepared Henry solution does not.[4,5] Chemical behavior as an assay of 2 in solution cannot be considered conclusive, however. Henry solution, Duden and Scharff solution, and formaldehyde–ammonia solutions of various molar ratios, as well as hexamine solutions, all react with nitrous acid at pH 1 to form 1,3,5-trinitroso-1,3,5-hexahydrotriazine (12) irreversibly in consistent yields of 25–30% based on methylene. The report by Wright[4] that his yield of 12 from Henry solution is 52% based on methylene is incorrect; calculated from the data which he reports, his correct yield is 26.6%; his yield from hexamine is 24%, as reported. The incorrect report is often cited in the secondary literature to support the high assay of 2 in Henry solution.[3] At higher pH (3–6) Henry solution as well as hexamine solutions react with nitrous acid to yield 3,7-dinitroso-1,3,5,7-tetraazabicyclo[3.3.1]nonane (13, 70–75%). As



12a 12b



13a (syn)

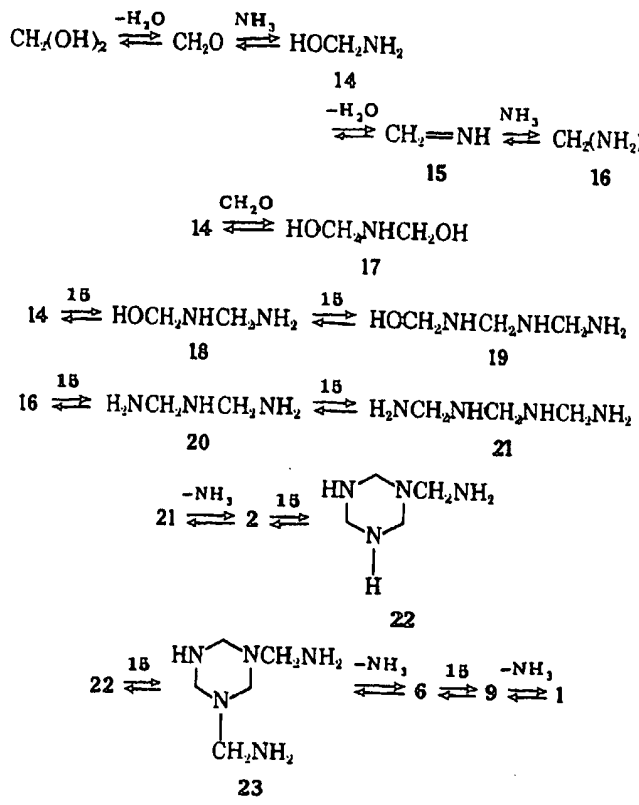


13b (anti)

explained by Bachmann,[15] the product distribution of 12 and 13 is dependent on the pH of the reaction medium. Their formation is an acid-catalyzed nitrosation of hexamine. Hexamine formation from 2 at pH 1 is evidently faster than nitrosation of 2 to form 12.

The high-resolution ^1H NMR spectrum of 12 in $(CD_3)_2CO$ reveals four methylene singlets, corresponding to a nearly statistical distribution of the symmetrical and asymmetrical forms 12a and 12b (1:3 ratio). The results are in agreement with the ^{13}C spectrum and an earlier report of proton spectra by Urbanski and co-workers, although their reported signals of the two forms were not completely resolved.[10c] The dinitroso compound 13 exhibits ^1H and ^{13}C NMR spectra at 30 °C corresponding to nearly equal amounts of syn and anti forms

Scheme I



13a and 13b, in agreement with previous reports[10a] (data in Table I).

Mechanism of Hexamine Formation. Mechanisms of hexamine formation from formaldehyde and ammonia have been discussed by others in several reports.[3-5,7] The present work and recent studies on aldehyde–ammonia and aldehyde–amine reactions, coupled with earlier information, now provide a better understanding of this complex reaction. For example, several extensive recent studies have elucidated detailed mechanisms of carbinolamine and imine formation.[16-21]

The kinetics of formaldehyde and ammonia consumption have been shown to be third order: first order in ammonia and second order in formaldehyde.[7] The data could be explained by a rate-limiting reaction of hemiaminal 14 with formaldehyde to form dimethylolamine (17, Scheme I). The pH–rate profile of this reaction reported by Kawasaki[7g,i] (maximum rate at pH 9.8) is of the same type observed by Abrams and Kallen for N-methylolation of amines.[19] The methylolation of ring-substituted anilines indicates hydronium, solvent, and hydroxide terms in the rate law; significant imine formation is not observed, nor is there a salt effect.[18] Abrams and Kallen also observed that the rate of monomethylolation was much faster than the rate of addition of the second *N*-methylol group. The ^{1}H NMR peak observed in the initial stage of the formaldehyde–ammonia reaction near δ 4.5 may be attributed to 17 (at 1 min elapsed reaction time, this signal appears as one intense, broad singlet, stronger in relative intensity than the one revealed in Figure 1 at 5 min).

The only kinetic study of the measured rate of hexamine formation from formaldehyde and ammonia is that of Winkler and co-workers.[7c,d] The rate law for this process differs from that of the initial consumption of reactants in being more complex. Formation of an unspecified byproduct was indicated by the data.

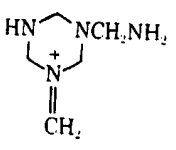
Scheme I describes an oversimplified mechanism for hexamine formation from formaldehyde and ammonia. It represents a summary of one possible reaction route which is in agreement with present knowledge. It is recognized that oxygen may be exchanged for nitrogen in some of the structures to represent additional species probably present. However, equilibria lead ultimately to heterocyclic products containing ring nitrogen only. For example, intermediates such as dimethylolamine (17) are probably consumed by retrogression to reactants rather than by direct participation in the reaction sequence(s). Methylenimine (15) is assumed to be the reactive aminomethylation intermediate, although its concentration is negligible relative to aminals 14 and 16.

Reaction intermediates of Scheme I are of acyclic and cyclic types. Acyclics include monomers (14, 15, 16), dimers (17, 18), and trimers (19, 20, 21). As discussed above, there exists kinetic and NMR evidence for hemiaminal monomer 14. The very labile homologous aldehyde ammonias, RCH(OH)NH$_2$, derived from alkanals have been isolated.[6] The instability and solubility of these are greatest when R is a small group (CH$_3$, C$_2$H$_5$). Dissociation to aldimines, RCH=NH, or retrogression to reactants occurs very readily; thus, the parent homologue (14) would be expected to be extremely reactive and labile. Acetaldimine (CH$_3$CH=NH), derived from CH$_3$CH(OH)NH$_2$, but not isolated, trimerizes very rapidly compared to its higher homologues;[6] owing to its low steady-state concentration, methylenimine (15) would be expected to be undetectable spectroscopically but to trimerize very rapidly to hexahydrotriazine 2.

Acyclic dimers 17 and 18 and trimer 19 could be represented by broad NCH$_2$O peaks seen near δ 4.5 (Figure 1). Good NMR and kinetic evidence for dimers CH$_3$CH(OH)NHCH(OH)CH$_3$ and CH$_3$CH(NH$_2$)NHCH(OH)CH$_3$ formed in the acetaldehyde–ammonia reaction has been reported.[22]

1,3,5-Hexahydrotriazine (2) represents the most abundant stable formaldehyde–ammonia reaction product other than hexamine (Figures 1 and 2). In the reaction of alkanals with ammonia, 2,4,6-trialkyl-1,3,5-hexahydrotriazines (3) are formed exclusively and in high yield.[1,6] Although labile compounds, they are more stable than 2 and do not react further with alkanal and ammonia to form *C*-alkyl derivatives of hexamine.

The linear pH dependence of the conversion rate 2 → . (Figure 4) suggests solvent-mediated proton-transfer steps. Although a detailed description of this process is not possible with present knowledge, a concerted route rather than discrete iminium ion participation is favored.[21,23] Ring closure of completely developed exocyclic iminium intermediates such as 24 → 6 and 25 → 1 would be sterically less favored than



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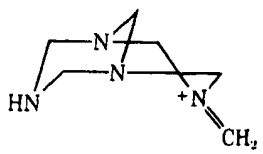

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nonplanar transition states. Similar acid-catalyzed processes thought to proceed through discrete methylene iminium ion intermediates, such as methylenebisamine formation,[24] and epimerization of 2,4,6-trialkyl-1,3,5-triazabicyclo[3.1.0]hexanes,[25] may also be concerted. The chemistry of methylene iminium salts has been reviewed.[26]

Near the completion of the reaction leading to 1 the solution becomes depleted in formaldehyde and monomers 14–16. These reactants, required for the subsequent conversion of intermediates to 1, are made available by acid-catalyzed retrogression of the intermediates to 15. Solutions containing 2 and ammonia are observed to react rapidly upon addition of formaldehyde, or acetic acid, to produce only hexamine as 2, 6, and other intermediates disappear. A rate-limiting, acid-catalyzed retrogression of 2 (to 15 and other products)

could thus account for the rate dependence observed in Figure 4. Ultimately, all intermediates are consumed to form 1, its retrogression rate in acidic medium being slower than any of its precursors.

Experimental Section

^1H NMR and ^{13}C NMR spectra were determined on a Varian XL-100 spectrometer with a Transform Technology TT-100 pulsed Fourier transform system. Chemical shift measurements were determined at ~30 °C unless otherwise stated and are referenced to sodium 3-(trimethylsilyl)propanesulfonate (H_2O or D_2O solutions).

Formaldehyde–Ammonium-d_4 Hydroxide-d Solution in D_2O. A mixture of 8.4 g of paraformaldehyde (95% assay), sodium bicarbonate (0.1 g), and D_2O (11.6 mL) was heated on the steam bath (~20 min) to obtain a clear solution. The solution contained 40% formaldehyde by weight as determined by reaction of an aliquot portion with dimedone.

Ammonia gas (5 g) was bubbled into 50 mL of D_2O at 0 °C. The resulting solution was distilled into 25 mL of D_2O until the total volume was 55 mL (6.0 M ammonium-d_4 hydroxide-d stock solution assayed by titration with 1 N NaOH solution).

The stock formaldehyde and ND_4OD solutions were diluted with D_2O and mixed as required to obtain various formaldehyde–ammonia ratios. ^1H and ^{13}C NMR spectra were determined at various intervals. Figure 1 shows ^1H spectra for a solution prepared from equal volumes of 1 M formaldehyde and ND_4OD solutions. Solutions were prepared in the same manner at various concentrations and various formaldehyde–ammonia ratios. No significant differences in NMR spectra were observed, although rates of hexamine formation were faster in dilute, formaldehyde-rich solutions (see Table II). A solution prepared (0.5 M in formaldehyde and NH_3) containing potassium carbonate (final concentration 0.07 M) revealed a rate of conversion to hexamine virtually unchanged from those containing no added potassium carbonate (Table II).

Proton-decoupled Fourier transform ^{13}C NMR spectra were determined at intervals at −10 °C since the reaction was too rapid at higher temperatures to accommodate the required signal-averaging times (500 scans, 15 min). The relative peak heights at various times for hexamine and 1,3,5-hexahydrotriazine, respectively, at −10 °C were the following: 2, 6 (1 h); 9, 15 (2.5 h); 11, 17 (3 h); 14, 18.5 (3.8 h); 15, 20 (4.5 h); 22, 14 (24 h). The spectrum obtained at 24 h is shown in Figure 2. The ^{13}C spin–lattice relaxation time, T_1, for aqueous hexamine, determined by the two-pulse inversion-recovery method, was found to be 0.65 s. This is sufficiently short to justify the assumption that the methylene carbon signal intensity is an accurate measure of hexamine concentration under the experimental conditions used (50° pulse repeated at 2.5-s intervals).[27]

Measurements of pH were determined at intervals on aliquot portions with a Beckman Model G glass electrode pH meter, calibrated with stock NH_4OH solutions. Peak heights of ^1H NMR spectra hexamine and triazine signals were also determined at intervals (from curves similar to those of Figure 1). As an arbitrary measure of the extent of reaction, a point was taken where the peak height ratio of hexamine to triazine was 4:1 (molar ratio = 2:1); pH was also determined at this point (pH was observed to be very constant near this point in the reaction). The pH of the solution was varied by initially adjusting the ammonia–formaldehyde ratio and reactant concentrations, or by addition of sodium hydroxide (Table II summarizes the data). A plot of pH vs. the log of the time at the occurrence of the specified peak height ratios is seen in Figure 4.

Henry Solution in H_2O. Samples of Henry solution were prepared by the procedure of Henry[12] as modified by Richmond, Myers, and Wright.[4] It is a clear, colorless liquid with a strong ammoniacal odor. ^1H and ^{13}C NMR spectra were determined at intervals on the original solution and on solutions diluted with an equal volume of D_2O. ^1H NMR spectra were determined at several temperatures between −10 and 80 °C. After 0.5 h at 80 °C or 3 h at 60 °C, all of the peaks except those of hexamine and water had disappeared.

A 1.0-g sample of freshly prepared Henry solution was concentrated to dryness at 5–15 °C (0.1 mm) to yield 0.5 g of white solid. Aliquot portions of this residue were extracted, separately, with D_2O, $CDCl_3$, CCl_4, and C_6D_6, and ^1H NMR spectra of the extracts were determined (discussion in text). Another aliquot was treated with concentrated sulfuric acid, followed by ignition in a crucible, to form an ash calculated to correspond to 0.18% potassium carbonate (~0.01 M in the original Henry solution).

Henry Solution in D_2O. A mixture of paraformaldehyde (3.0 g, 0.1 mol), D_2O (4.5 mL), and sodium bicarbonate (0.1 g) was heated on the steam bath until a clear solution resulted (~20 min). After chilling to 0 °C, ammonia gas (1.7 g, 0.1 mol) was passed into the solution during 15 min, keeping the temperature below 10 °C. Potassium carbonate (5 g) was added in portions with stirring during 10 min, keeping the temperature below 3 °C. After standing at 0 °C for 20 min, the top layer (4.3 g) was separated and stored at 0 °C. ^1H NMR spectra of an aliquot sample stored at 25 °C were determined at intervals. Peak heights of signals for hexamine (1, δ 4.75), 1,3,5-hexahydrotriazine (2, δ 3.95), and 1,3,5,7-tetraazabicyclo[3.3.1]nonane (3, δ 3.86; nonbridgehead methylenes only) were determined at intervals. The peak heights were each divided by the number of methylene groups corresponding to the signal, 6, 3, and 4, respectively, in order to obtain the relative molar concentration of each of the three components. Data are plotted in Figure 3. The Henry solution–D_2O solvent NMR spectra were similar to those of the Henry solution–H_2O solvent except for a weaker H_2O signal. Changing the original ratio of reactants formaldehyde/ammonia from 1:1 to 3:2 did not significantly alter the spectra of the final solution.

Duden and Scharff Solution in D_2O. Solution A: paraformaldehyde (3.16 g, 95% assay, 0.1 mol), sodium bicarbonate (0.1 g), and D_2O (4.5 g) were warmed on the steam bath until a clear solution resulted (about 20 min). Solution B: to a solution of ammonium chloride (5.35 g, 0.1 mol) in 20 mL of warm D_2O was added 4.4 g (0.11 mol) of sodium hydroxide. A portion of solution A (0.75 g, 0.01 mol of formaldehyde) was added slowly to a portion of solution B (3.2 g, 0.01 mol of ammonium chloride and 0.011 mol of sodium hydroxide) with ice-bath cooling, keeping the temperature below 15 °C during the addition. NMR and pH measurements were determined at intervals (data are in Table II). Owing to the high sodium ion concentration of the solution (2.8 M), accurate pH measurements could not be made with the glass electrode employed; a group of pH indicator papers was used. A "synthetic" Duden and Scharff solution was prepared by addition of 0.75 g of formaldehyde solution A, above (0.01 mol of formaldehyde), to a solution containing 1.56 mL of 6.4 M ND_4OD in D_2O, 1.0 mL of 0.1 M NaOD in D_2O, and 0.585 g of NaCl. Its properties, as expected, were identical with those of the Duden and Scharff solution prepared above.

1,3,5-Trinitroso-1,3,5-hexahydrotriazine (12). A mixture of paraformaldehyde (4.74 g, 95% assay, 0.15 mol), water (9.0 mL), and sodium bicarbonate (0.05 g) was heated on the steam bath to obtain a clear solution. To the solution, chilled to −10 °C in an ice–salt bath, was added cold concentrated ammonium hydroxide (10.0 mL, 0.15 mol) with stirring, keeping the temperature below 0 °C during the addition (15 min). After being stored at 0 °C for 6 h, the solution was poured into 200 mL of cold water (3 °C). To this solution was added simultaneously a solution of sodium nitrite (17.8 g, 97% assay, 0.25 mol) in 50 mL of water and a second solution of concentrated sulfuric acid (8.4 mL) in 50 mL of water with stirring, keeping the temperature below 4 °C; the sulfuric solution was added fast enough to keep the solution at pH 1 throughout the addition period (total time 20 min). After addition was complete, the mixture was stirred at 3 °C for 45 min; filtration, followed by washing with water, gave 2.53 g (29%) of pure 12, mp 105–106 °C (lit.[15] mp 106–107 °C), identical with an authentic sample. The ^1H and ^{13}C NMR spectra of the product [$(CD_3)_2CO$ solvent] revealed the absence of impurities, including 13 (data are in Table I).

Registry No.—1, 100-97-0; 2, 110-90-7; 3a, 638-14-2; 4, 14558-49-7; 5, 505-21-5; 6, 281-19-6; 7, 949-56-4; 8, 69470-04-8; 9, 69470-05-9; 12, 13980-04-6; 13, 101-25-7; formaldehyde, 50-00-0; ammonia, 7664-41-7; nitrous acid, 7782-77-6.

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Synthesis, Stereochemistry, and Rearrangement of 9-Alkylthioxanthene *N*-(*p*-Toluenesulfonyl)sulfilimines

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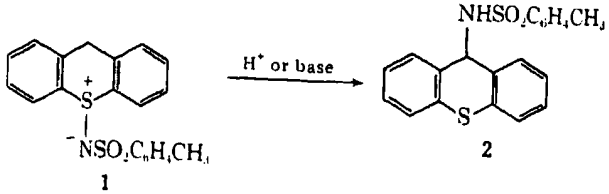
cis- and *trans*-9-Methyl- (**6a**), *cis*- and *trans*-9-ethyl- (**6b**), and *trans*-9-isopropylthioxanthene *N*-(*p*-toluenesulfonyl)sulfilimines (**6c**) were synthesized by two routes: (i) tosylation of 10-aminothioxanthenium mesitylenesulfonates, which were prepared by the reaction of the corresponding thioxanthenes with *O*-mesitylenesulfonylhydroxylamine; and (ii) reaction of the thioxanthenes with chloramine T. The stereochemistry of the sulfilimines **6a–c** was ascertained by a comparison of the NMR spectra of **6a–c** with those of the corresponding sulfoxides, whose stereochemistry has been well established, and by the thermal equilibration of **6a–c**. When refluxed in dioxane containing small amounts of concentrated hydrochloric acid, **6a–c** were reduced to the corresponding thioxanthenes. Upon treatment with DBU in benzene *cis*- and *trans*-**6a,b** and *trans*-**6c** rearranged to the corresponding 9-alkyl-9-(*N*-*p*-toluenesulfonamido)thioxanthenes. The rates of the rearrangement decreased in the order *trans*-**6a** > *trans*-**6b** > *cis*-**6a** > *cis*-**6b** > *trans*-**6c**.

Thioxanthene *N*-(*p*-toluenesulfonyl)sulfilimine (**1**) undergoes acid- and base-catalyzed rearrangement to 9-*N*-(*p*-toluenesulfonamido)thioxanthene (**2**).[1,2] We have now examined the effect of the 9-alkyl substituents on this rearrangement. In this paper the synthesis and stereochemistry of 9-alkylthioxanthene *N*-(*p*-toluenesulfonyl)sulfilimines (**6a–c**), and their behavior toward acid and base, are described.



1 2

Results and Discussion

Synthesis. 9-Alkylthioxanthene *N*-(*p*-toluenesulfonyl)-sulfilimines (**6a–c**) were synthesized by two routes as shown in Scheme I: (method A) tosylation of 10-aminothioxanthenium mesitylenesulfonates (**4a–c**),[3] which were prepared by the reaction of the thioxanthenes **3a–c** with *O*-mesitylenesulfonylhydroxylamine (MSH);[4] and (method B) reaction of **3a–c** with chloramine T.

Treatment of the thioxanthenes **3a–c** with 1 equiv of MSH in methylene chloride at room temperature gave the corresponding *S*-amine salts **4a–c**. Thus, 9-methylthioxanthene (**3a**) afforded two isomeric *S*-amine salts **4a** in a cis/trans ratio of ~3:5 (by NMR spectroscopy), which could be separated by fractional recrystallization. Tosylation of each isomer gave pure *cis*- and *trans*-**6a** in 8 and 19% overall yields, respectively. 9-Ethylthioxanthene (**3b**) also gave a mixture of cis and trans isomers of the *S*-amine salts **4b**. This mixture was directly converted into two isomeric *N*-(*p*-toluenesulfonyl)-sulfilimines **6b**, which were separated by column chromatography to give pure *cis*- and *trans*-**6b** in 9 and 31% overall yields, respectively. 9-Isopropylthioxanthene (**3c**) produced exclusively the trans isomer of the *S*-amine salt **4c** in 73% yield. Passing an ethanolic solution of *trans*-**4c** through a

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Antiseptics and Disinfectants: Activity, Action, and Resistance

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INTRODUCTION

Antiseptics and disinfectants are used extensively in hospitals and other health care settings for a variety of topical and hard-surface applications. In particular, they are an essential part of infection control practices and aid in the prevention of nosocomial infections (277, 454). Mounting concerns over the potential for microbial contamination and infection risks in the food and general consumer markets have also led to increased use of antiseptics and disinfectants by the general public. A wide variety of active chemical agents (or "biocides") are found in these products, many of which have been used for hundreds of years for antisepsis, disinfection, and preservation (39). Despite this, less is known about the mode of action of these active agents than about antibiotics. In general, biocides have a broader spectrum of activity than antibiotics, and, while antibiotics tend to have specific intracellular targets, biocides may have multiple targets. The widespread use of antiseptic and disinfectant products has prompted some speculation on the development of microbial resistance, in particular cross-resistance to antibiotics. This review considers what is known about the mode of action of, and mechanisms of microbial resistance to, antiseptics and disinfectants and attempts, wherever possible, to relate current knowledge to the clinical environment.

A summary of the various types of biocides used in antiseptics and disinfectants, their chemical structures, and their clinical uses is shown in Table 1. It is important to note that many of these biocides may be used singly or in combination in a variety of products which vary considerably in activity against microorganisms. Antimicrobial activity can be influenced by many factors such as formulation effects, presence of an organic load, synergy, temperature, dilution, and test method. These issues are beyond the scope of this review and are discussed elsewhere (123, 425, 444, 446, 451).

DEFINITIONS

"Biocide" is a general term describing a chemical agent, usually broad spectrum, that inactivates microorganisms. Because biocides range in antimicrobial activity, other terms may be more specific, including "-static," referring to agents which inhibit growth (e.g., bacteriostatic, fungistatic, and sporistatic) and "-cidal," referring to agents which kill the target organism (e.g., sporicidal, virucidal, and bactericidal). For the purpose of this review, antibiotics are defined as naturally occurring or synthetic organic substances which inhibit or destroy selective bacteria or other microorganisms, generally at low concentrations; antiseptics are biocides or products that destroy or inhibit the growth of microorganisms in or on living tissue (e.g. health care personnel handwashes and surgical scrubs); and disinfectants are similar but generally are products or biocides that are used on inanimate objects or surfaces. Disinfectants can be sporostatic but are not necessarily sporicidal.

Sterilization refers to a physical or chemical process that completely destroys or removes all microbial life, including spores. Preservation is the prevention of multiplication of microorganisms in formulated products, including pharmaceuticals and foods. A number of biocides are also used for cleaning purposes; cleaning in these cases refers to the physical removal of foreign material from a surface (40).

MECHANISMS OF ACTION

Introduction

Considerable progress has been made in understanding the mechanisms of the antibacterial action of antiseptics and disinfectants (215, 428, 437). By contrast, studies on their modes of action against fungi (426, 436), viruses (298, 307), and protozoa (163) have been rather sparse. Furthermore, little is known about the means whereby these agents inactivate prions (503).

Whatever the type of microbial cell (or entity), it is probable that there is a common sequence of events. This can be envisaged as interaction of the antiseptic or disinfectant with the cell surface followed by penetration into the cell and action at the target site(s). The nature and composition of the surface vary from one cell type (or entity) to another but can also alter as a result of changes in the environment (57, 59). Interaction at the cell surface can produce a significant effect on viability (e.g. with glutaraldehyde) (374, 421), but most antimicrobial agents appear to be active intracellularly (428, 451). The outermost layers of microbial cells can thus have a significant effect on their susceptibility (or insusceptibility) to antiseptics and disinfectants; it is disappointing how little is known about the passage of these antimicrobial agents into different types of microorganisms. Potentiation of activity of most biocides may be achieved by the use of various additives, as shown in later parts of this review.

In this section, the mechanisms of antimicrobial action of a range of chemical agents that are used as antiseptics or disinfectants or both are discussed. Different types of microorganisms are considered, and similarities or differences in the nature of the effect are emphasized. The mechanisms of action are summarized in Table 2.

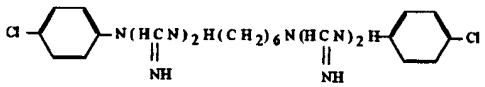
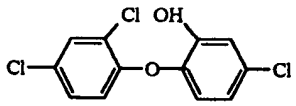
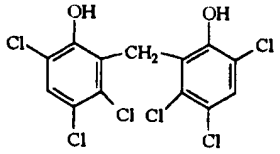
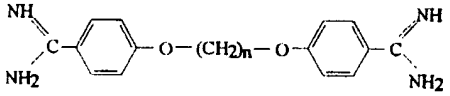
General Methodology

A battery of techniques are available for studying the mechanisms of action of antiseptics and disinfectants on microorganisms, especially bacteria (448). These include examination of uptake (215, 428, 459), lysis and leakage of intracellular constituents (122), perturbation of cell homeostasis (266, 445), effects on model membranes (170), inhibition of enzymes, electron transport, and oxidative phosphorylation (162, 272), interaction with macromolecules (448, 523), effects on macromolecular biosynthetic processes (133), and microscopic examination of biocide-exposed cells (35). Additional and useful information can be obtained by calculating concentration exponents (*n* values [219, 489]) and relating these to membrane activity (219). Many of these procedures are valuable for detecting and evaluating antiseptics or disinfectants used in combination (146, 147, 202, 210).

Similar techniques have been used to study the activity of antiseptics and disinfectants against fungi, in particular yeasts. Additionally, studies on cell wall porosity (117–119) may provide useful information about intracellular entry of disinfectants and antiseptics (204–208).

Mechanisms of antiprotozoal action have not been widely investigated. One reason for this is the difficulty in culturing some protozoa (e.g., *Cryptosporidium*) under laboratory conditions. However, the different life stages (trophozoites and cysts) do provide a fascinating example of the problem

TABLE 1. Chemical structures and uses of biocides in antiseptics and disinfectants

Alcohols	Ethanol	$CH_3—CHOH$	Antisepsis
	Isopropanol	$CH_3(CH_3)CHOH$	Disinfection
			Preservation
Aldehydes	Glutaraldehyde	$OH—CCH_2CH_2CH_2C—HO$	Disinfection
	Formaldehyde	$H—C—HO$	Sterilization
			Preservation
Anilides	General structure	$C_6H_5.NH.COR$	Antisepsis
	Triclocarban		
Biguanides	Chlorhexidine		Antisepsis
			Antiplaque agents
	Alexidine, polymeric biguanides		Preservation
			Disinfection
Bisphenols	Triclosan		Antisepsis
			Antiplaque agents
	Hexachlorophene		Deodorants
			Preservation
Diamidines	Propamidine		Antisepsis
			Preservation
	Dibromopropamidine		

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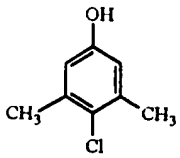
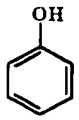
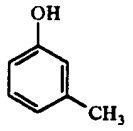
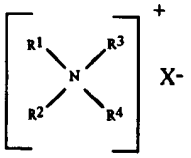
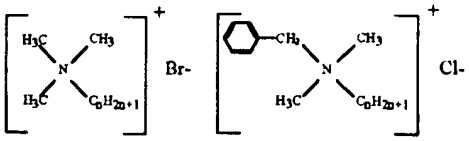
of how changes in cytology and physiology can modify responses to antiseptics and disinfectants. Khunkitti et al. (251–255) have explored this aspect by using indices of viability, leakage, uptake, and electron microscopy as experimental tools.

Some of these procedures can also be modified for studying effects on viruses and phages (e.g., uptake to whole cells and viral or phage components, effects on nucleic acids and proteins, and electron microscopy) (401). Viral targets are

TABLE 1—*Continued*

Halogen-releasing agents	Chlorine compounds	◇OCl-, HOCl, Cl_2	Disinfection Antisepsis
	Iodine compounds	◇I_2	Cleaning
Halophenols	Chloroxylenol (PCMX)		Antisepsis Preservation
Heavy metal derivatives	Silver compounds	Ag	Preservation Antisepsis
	Mercury compounds	Hg	Disinfection
Peroxygens	Hydrogen peroxide	H_2O_2	Disinfection
	Ozone	O_3	Sterilization
	Peracetic acid	$CH_3.COOOH$	
Phenols and cresols	Phenol		Disinfection Preservation
	Cresol		
Quaternary ammonium compounds	General structure		Disinfection Antisepsis Preservation
	Cetrimide, benzalkonium chloride		Cleaning

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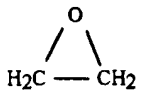
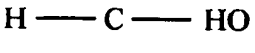
predominantly the viral envelope (if present), derived from the host cell cytoplasmic or nuclear membrane; the capsid, which is responsible for the shape of virus particles and for the protection of viral nucleic acid; and the viral genome. Release of an intact viral nucleic acid into the environment following capsid destruction is of potential concern since some nucleic acids are infective when liberated from the capsid (317), an aspect that must be considered in viral disinfection. Important considerations in viral inactivation are dealt with by Klein and Deforest (259) and Prince et al.

TABLE 1—*Continued*

Vapor phase	Ethylene oxide		Sterilization Disinfection
	Formaldehyde		
	Hydrogen peroxide	H_2O_2	

(384), while an earlier paper by Grossgebauer is highly recommended (189).

Alcohols

Although several alcohols have been shown to be effective antimicrobials, ethyl alcohol (ethanol, alcohol), isopropyl alcohol (isopropanol, propan-2-ol) and *n*-propanol (in particular in Europe) are the most widely used (337). Alcohols exhibit rapid broad-spectrum antimicrobial activity against vegetative bacteria (including mycobacteria), viruses, and fungi but are not sporicidal. They are, however, known to inhibit sporulation and spore germination (545), but this effect is reversible (513). Because of the lack of sporicidal activity, alcohols are not recommended for sterilization but are widely used for both hard-surface disinfection and skin antisepsis. Lower concentrations may also be used as preservatives and to potentiate the activity of other biocides. Many alcohol products include low levels of other biocides (in particular chlorhexidine), which remain on the skin following evaporation of the alcohol, or excipients (including emollients), which decrease the evaporation time of the alcohol and can significantly increase product efficacy (68). In general, isopropyl alcohol is considered slightly more efficacious against bacteria (95) and ethyl alcohol is more potent against viruses (259); however, this is dependent on the concentrations of both the active agent and the test microorganism. For example, isopropyl alcohol has greater lipophilic properties than ethyl alcohol and is less active against hydrophilic viruses (e.g., poliovirus) (259). Generally, the antimicrobial activity of alcohols is significantly lower at concentrations below 50% and is optimal in the 60 to 90% range.

Little is known about the specific mode of action of alcohols, but based on the increased efficacy in the presence of water, it is generally believed that they cause membrane damage and rapid denaturation of proteins, with subsequent interference with metabolism and cell lysis (278, 337). This is supported by specific reports of denaturation of *Escherichia coli* dehydrogenases (499) and an increased lag phase in *Enterobacter aerogenes*, speculated to be due to inhibition of metabolism required for rapid cell division (101).

Aldehydes

Glutaraldehyde. Glutaraldehyde is an important dialdehyde that has found usage as a disinfectant and sterilant, in particular for low-temperature disinfection and sterilization of endoscopes and surgical equipment and as a fixative in electron

TABLE 2. Summary of mechanisms of antibacterial action of antiseptics and disinfectants

Target	Antiseptic or disinfectant	Mechanism of action
Cell envelope (cell wall, outer membrane)	Glutaraldehyde	Cross-linking of proteins
	EDTA, other permeabilizers	Gram-negative bacteria: removal of Mg^{2+}, release of some LPS
Cytoplasmic (inner) membrane	QACs	Generalized membrane damage involving phospholipid bilayers
	Chlorhexidine	Low concentrations affect membrane integrity, high concentrations cause congealing of cytoplasm
	Diamines	Induction of leakage of amino acids
	PHMB, alexidine	Phase separation and domain formation of membrane lipids
	Phenols	Leakage; some cause uncoupling
Cross-linking of macromolecules	Formaldehyde	Cross-linking of proteins, RNA, and DNA
	Glutaraldehyde	Cross-linking of proteins in cell envelope and elsewhere in the cell
DNA intercalation	Acridines	Intercalation of an acridine molecule between two layers of base pairs in DNA
Interaction with thiol groups	Silver compounds	Membrane-bound enzymes (interaction with thiol groups)
Effects on DNA	Halogens	Inhibition of DNA synthesis
	Hydrogen peroxide, silver ions	DNA strand breakage
Oxidizing agents	Halogens	Oxidation of thiol groups to disulfides, sulfoxides, or disulfoxides
	Peroxygens	Hydrogen peroxide: activity due to from formation of free hydroxy radicals ('OH), which oxidize thiol groups in enzymes and proteins; PAA: disruption of thiol groups in proteins and enzymes

TABLE 3. Mechanism of antimicrobial action of glutaraldehyde

Target microorganism	Glutaraldehyde action
Bacterial spores	Low concentrations inhibit germination; high concentrations are sporicidal, probably as a consequence of strong interaction with outer cell layers
Mycobacteria	Action unknown, but probably involves mycobacterial cell wall
Other nonsporulating bacteria	Strong association with outer layers of gram-positive and gram-negative bacteria; cross-linking of amino groups in protein; inhibition of transport processes into cell
Fungi	Fungal cell wall appears to be a primary target site, with postulated interaction with chitin
Viruses	Actual mechanisms unknown, but involve protein-DNA cross-links and capsid changes
Protozoa	Mechanism of action not known

icroscopy. Glutaraldehyde has a broad spectrum of activity against bacteria and their spores, fungi, and viruses, and a considerable amount of information is now available about the ways whereby these different organisms are inactivated (Tables 2 and 3). Earlier reviews of its mechanisms of action have been published (179, 182, 374, 482).

The first reports in 1964 and 1965 (182) demonstrated that glutaraldehyde possessed high antimicrobial activity. Subsequently, research was undertaken to evaluate the nature of its bactericidal (339–344, 450) and sporicidal (180, 181, 507, 508) action. These bactericidal studies demonstrated (374) a strong binding of glutaraldehyde to outer layers of organisms such as *E. coli* and *Staphylococcus aureus* (179, 212, 339–341, 343, 344), inhibition of transport in gram-negative bacteria (179), inhibition of dehydrogenase activity (343, 344) and of periplasmic enzymes (179), prevention of lysostaphin-induced lysis in *S. aureus* (453) and of sodium lauryl sulfate-induced lysis in *E. coli* (340, 344), inhibition of spheroplast and protoplast lysis in hypotonic media (340, 344), and inhibition of RNA, DNA, and protein synthesis (320). Strong interaction of glutaraldehyde with lysine and other amino acids has been demonstrated (450).

Clearly, the mechanism of action of glutaraldehyde involves a strong association with the outer layers of bacterial cells, specifically with unprotonated amines on the cell surface, possibly representing the reactive sites (65). Such an effect could explain its inhibitory action on transport and on enzyme systems, where access of substrate to enzyme is prohibited. Partial or entire removal of the cell wall in hypertonic medium, leading to the production of spheroplasts or protoplasts and the subsequent prevention of lysis by glutaraldehyde when these forms are diluted in a hypotonic environment, suggests an additional effect on the inner membrane, a finding substantiated by the fact that the dialdehyde prevents the selective release of some membrane-bound enzymes of *Micrococcus lysodeikticus* (138). Glutaraldehyde is more active at alkaline than at acidic pHs. As the external pH is altered from acidic to alkaline, more reactive sites will be formed at the cell surface, leading to a more rapid bactericidal effect. The cross-links thus obtained mean that the cell is then unable to undertake most, if not all, of its essential functions. Glutaraldehyde is also mycobactericidal. Unfortunately, no critical studies have as yet been undertaken to evaluate the nature of this action (419).

The bacterial spore presents several sites at which interaction with glutaraldehyde is possible, although interaction with a particular site does not necessarily mean that this is associated with spore inactivation. *E. coli*, *S. aureus*, and vegetative cells of *Bacillus subtilis* bind more glutaraldehyde than do resting spores of *B. subtilis* (377, 378); uptake of glutaraldehyde is greater during germination and outgrowth than with mature spores but still lower than with vegetative cells. Low concentrations of the dialdehyde (0.1%) inhibit germination, whereas much higher concentrations (2%) are sporicidal. The aldehyde, at both acidic and alkaline pHs, interacts strongly with the outer spore layers (508, 509); this interaction reduces the release of dipicolinic acid (DPA) from heated spores and the lysis induced by mercaptoethanol (or thioglycolate)-peroxide combinations. Low concentrations of both acidic and alkaline glutaraldehyde increase the surface hydrophobicity of spores, again indicating an effect at the outermost regions of the cell. It has been observed by various authors (182, 374, 376, 380) that the greater sporicidal activity of glutaraldehyde at alkaline pH is not reflected by differences in uptake; however, uptake per se reflects binding and not necessarily penetration into the spore. It is conceivable that acidic glutaraldehyde interacts with and remains at the cell surface whereas alkaline glutaraldehyde penetrates more deeply into the spore. This contention is at odds with the hypothesis of Bruch (65), who envisaged the acidic form penetrating the coat and reacting with the cortex while the alkaline form attacked the coat, thereby destroying the ability of the spore to function solely as a result of this surface phenomenon. There is, as yet, no evidence to support this theory. Novel glutaraldehyde formulations based on acidic rather than alkaline glutaraldehyde, which benefit from the greater inherent stability of the aldehyde at lower pH, have been produced. The improved sporicidal activity claimed for these products may be obtained by agents that potentiate the activity of the dialdehyde (414, 421).

During sporulation, the cell eventually becomes less susceptible to glutaraldehyde (see "Intrinsic resistance of bacterial spores"). By contrast, germinating and outgrowing cells reacquire sensitivity. Germination may be defined as an irreversible process in which there is a change of an activated spore from a dormant to a metabolically active state within a short period. Glutaraldehyde exerts an early effect on the germination process. L-Alanine is considered to act by binding to a specific receptor on the spore coat, and once spores are triggered to germinate, they are committed irreversibly to losing their dormant properties (491). Glutaraldehyde at high concentrations inhibits the uptake of L-[^{14}C]alanine by *B. subtilis* spores, albeit by an unknown mechanism (379, 414). Glutaraldehyde-treated spores retain their refractivity, having the same appearance under the phase-contrast microscope as normal, untreated spores even when the spores are subsequently incubated in germination medium. Glutaraldehyde is normally used as a 2% solution to achieve a sporicidal effect (16, 316); low concentrations (<0.1%) prevent phase darkening of spores and also prevent the decrease in optical density associated with a late event in germination. By contrast, higher concentrations (0.1 to 1%) significantly reduce the uptake of L-alanine, possibly as a result of a sealing effect of the aldehyde on the cell surface. Mechanisms involved in the revival of glutaraldehyde-treated spores are discussed below (see "Intrinsic resistance of bacterial spores").

There are no recent studies of the mechanisms of fungicidal action of glutaraldehyde. Earlier work had suggested that the fungal cell wall was a major target site (179, 182, 352), especially the major wall component, chitin, which is analogous to the peptidoglycan found in bacterial cell walls.

Glutaraldehyde is a potent virucidal agent (143, 260). It reduces the activity of hepatitis B surface antigen (HBsAg) and especially hepatitis B core antigen ([HBcAg] in hepatitis B virus [HBV]) (3) and interacts with lysine residues on the surface of hepatitis A virus (HAV) (362). Low concentrations

(<0.1%) of alkaline glutaraldehyde are effective against purified poliovirus, whereas poliovirus RNA is highly resistant to aldehyde concentrations up to 1% at pH 7.2 and is only slowly inactivated at pH 8.3 (21). In other words, the complete poliovirus particle is much more sensitive than poliovirus RNA. In light of this, it has been inferred that glutaraldehyde-induced loss of infectivity is associated with capsid changes (21). Glutaraldehyde at the low concentrations of 0.05 and 0.005% interacts with the capsid proteins of poliovirus and echovirus, respectively; the differences in sensitivity probably reflect major structural variations in the two viruses (75).

Bacteriophages were recently studied to obtain information about mechanisms of virucidal action (298–304, 306, 307). Many glutaraldehyde-treated *P. aeruginosa* F116 phage particles had empty heads, implying that the phage genome had been ejected. The aldehyde was possibly bound to F116 double-stranded DNA but without affecting the molecule; glutaraldehyde also interacted with phage F116 proteins, which were postulated to be involved in the ejection of the nucleic acid. Concentrations of glutaraldehyde greater than 0.1 to 0.25% significantly affected the transduction of this phage; the transduction process was more sensitive to the aldehyde than was the phage itself. Glutaraldehyde and other aldehydes were tested for their ability to form protein-DNA cross-links in simian virus 40 (SV40); aldehydes (i.e., glyoxal, furfural, prionaldehyde, acetaldehyde, and benzylaldehyde) without detectable cross-linking ability had no effect on SV40 DNA synthesis, whereas acrolein, glutaraldehyde, and formaldehyde, which formed such cross-links (144, 271, 297), inhibited DNA synthesis (369).

Formaldehyde. Formaldehyde (methanal, CH_2O) is a monoaldehyde that exists as a freely water-soluble gas. Formaldehyde solution (formalin) is an aqueous solution containing *ca.* 34 to 38% (wt/wt) CH_2O with methanol to delay polymerization. Its clinical use is generally as a disinfectant and sterilant in liquid or in combination with low-temperature steam. Formaldehyde is bactericidal, sporicidal, and virucidal, but it works more slowly than glutaraldehyde (374, 482).

Formaldehyde is an extremely reactive chemical (374, 442) that interacts with protein (156, 157), DNA (155), and RNA (155) in vitro. It has long been considered to be sporicidal by virtue of its ability to penetrate into the interior of bacterial spores (500). The interaction with protein results from a combination with the primary amide as well as with the amino groups, although phenol groups bind little formaldehyde (155). It has been proposed that formaldehyde acts as a mutagenic agent (291) and as an alkylating agent by reaction with carboxyl, sulfhydryl, and hydroxyl groups (371). Formaldehyde also reacts extensively with nucleic acid (489) (e.g., the DNA of bacteriophage T2) (190). As pointed out above, it forms protein-DNA cross-links in SV40, thereby inhibiting DNA synthesis (369). Low concentrations of formaldehyde are sporostatic and inhibit germination (512). Formaldehyde alters HBsAg and HBcAg of HBV (3).

It is difficult to pinpoint accurately the mechanism(s) responsible for formaldehyde-induced microbial inactivation. Clearly, its interactive, and cross-linking properties must play a considerable role in this activity. Most of the other aldehydes (glutaraldehyde, glyoxyl, succinaldehyde, and *o*-phthalaldehyde [OPA]) that have sporicidal activity are dialdehydes (and of these, glyoxyl and succinaldehyde are weakly active). The distance between the two aldehyde groups in glutaraldehyde (and possibly in OPA) may be optimal for interaction of these-CHO groups in nucleic acids and especially in proteins and enzymes (428).

Formaldehyde-releasing agents. Several formaldehyde-releasing agents have been used in the treatment of peritonitis (226, 273). They include noxythiolin (oxymethylenethiourea), tauroline (a condensate of two molecules of the aminosulponic acid taurine with three molecules of formaldehyde), hexamine (hexamethylenetetramine, methenamine), the resins melamine and urea formaldehydes, and imidazolone derivatives such as dantoin. All of these agents are claimed to be microbicidal on account of the release of formaldehyde. However, because the antibacterial activity of taurolin is greater than that of free formaldehyde, the activity of taurolin is not entirely the result of formaldehyde action (247).

TABLE 4. Mechanisms of antimicrobial action of chlorhexidine

Type of microorganism	Chlorhexidine action
Bacterial spores	Not sporicidal but prevents development of spores; inhibits spore outgrowth but not germination
Mycobacteria	Mycobacteristatic (mechanism unknown) but not mycobactericidal
Other nonsporulating bacteria	Membrane-active agent, causing protoplast and spheroplast lysis; high concentrations cause precipitation of proteins and nucleic acids
Yeasts	Membrane-active agent, causing protoplast lysis and intracellular leakage; high concentrations cause intracellular coagulation
Viruses	Low activity against many viruses; lipid-enveloped viruses more sensitive than nonenveloped viruses; effect possibly on viral envelope, perhaps the lipid moieties
Protozoa	Recent studies against *A. castellanii* demonstrate membrane activity (leakage) toward trophozoites, less toward cysts

***o*-Phthalaldehyde.** OPA is a new type of disinfectant that is claimed to have potent bactericidal and sporicidal activity and has been suggested as a replacement for glutaraldehyde in endoscope disinfection (7). OPA is an aromatic compound with two aldehyde groups. To date, the mechanism of its antimicrobial action has been little studied, but preliminary evidence (526) suggests an action similar to that of glutaraldehyde. Further investigations are needed to corroborate this opinion.

Anilides

The anilides have been investigated primarily for use as antiseptics, but they are rarely used in the clinic. Triclocarban (TCC; 3,4,4′-triclorocarbanilide) is the most extensively studied in this series and is used mostly in consumer soaps and deodorants. TCC is particularly active against gram-positive bacteria but significantly less active against gram-negative bacteria and fungi (30) and lacks appreciable substantivity (persistency) for the skin (37). The anilides are thought to act by adsorbing to and destroying the semipermeable character of the cytoplasmic membrane, leading to cell death (194).

Biguanides

Chlorhexidine. Chlorhexidine is probably the most widely used biocide in antiseptic products, in particular in handwashing and oral products but also as a disinfectant and preservative. This is due in particular to its broad-spectrum efficacy, substantivity for the skin, and low irritation. Of note, irritability has been described and in many cases may be product specific (167, 403). Despite the advantages of chlorhexidine, its activity is pH dependent and is greatly reduced in the presence of organic matter (430). A considerable amount of research has been undertaken on the mechanism of the antimicrobial action of this important bisbiguanide (389) (Tables 2 and 4), although most of the attention has been devoted to the way in which it

inactivates nonsporulating bacteria (215, 428, 430, 431, 451). Nevertheless, sufficient data are now available to examine its sporostatic and mycobacteriostatic action, its effects on yeasts and protozoa, and its antiviral activity.

Chlorhexidine is a bactericidal agent (120, 215). Its interaction and uptake by bacteria were studied initially by Hugo et al. (222–224), who found that the uptake of chlorhexidine by *E. coli* and *S. aureus* was very rapid and depended on the chlorhexidine concentration and pH. More recently, by using [^{14}C]chlorhexidine gluconate, the uptake by bacteria (145) and yeasts (204) was shown to be extremely rapid, with a maximum effect occurring within 20 s. Damage to the outer cell layers takes place (139) but is insufficient to induce lysis or cell death. The agent then crosses the cell wall or outer membrane, presumably by passive diffusion, and subsequently attacks the bacterial cytoplasmic or inner membrane or the yeast plasma membrane. In yeasts, chlorhexidine "partitions" into the cell wall, plasma membrane, and cytoplasm of cells (205). Damage to the delicate semipermeable membrane is followed by leakage of intracellular constituents, which can be measured by appropriate techniques. Leakage is not per se responsible for cellular inactivation but is a consequence of cell death (445). High concentrations of chlorhexidine cause coagulation of intracellular constituents. As a result, the cytoplasm becomes congealed, with a consequent reduction in leakage (222–224, 290), so that there is a biphasic effect on membrane permeability. An initial high rate of leakage rises as the concentration of chlorhexidine increases, but leakage is reduced at higher biocide concentrations because of the coagulation of the cytosol.

Chlorhexidine was claimed by Harold et al. (199) to be an inhibitor of both membrane-bound and soluble ATPase as well as of net K^+ uptake in *Enterococcus faecalis*. However, only high biguanide concentrations inhibit membrane-bound ATPase (83), which suggests that the enzyme is not a primary target for chlorhexidine action. Although chlorhexidine collapses the membrane potential, it is membrane disruption rather than ATPase inactivation that is associated with its lethal effects (24, 272).

The effects of chlorhexidine on yeast cells are probably similar to those previously described for bacteria (204–207). Chlorhexidine has a biphasic effect on protoplast lysis, with reduced lysis at higher biguanide concentrations. Furthermore, in whole cells, the yeast cell wall may have some effect in limiting the uptake of the biguanide (208). The findings presented here and elsewhere (47, 136, 137, 527) demonstrate an effect on the fungal plasma membrane but with significant actions elsewhere in the cell (47). Increasing concentrations of chlorhexidine (up to 25 μg/ml) induce progressive lysis of *Saccharomyces cerevisiae* protoplasts, but higher biguanide concentrations result in reduced lysis (205).

Work to date suggests that chlorhexidine has a similar effect on the trophozoites of *Acanthameoba castellanii*, with the cysts being less sensitive (251–255). Furr (163) reviewed the effects of chlorhexidine and other biocides on *Acanthameoba* and showed that membrane damage in these protozoa is a significant factor in their inactivation.

Mycobacteria are generally highly resistant to chlorhexidine (419). Little is known about the uptake of chlorhexidine (and other antiseptics and disinfectants) by mycobacteria and on the biochemical changes that occur in the treated cells. Since the MICs for some mycobacteria are on the order of those for chlorhexidine-sensitive, gram-positive cocci (48), the inhibitory effects of chlorhexidine on mycobacteria may not be dissimilar to those on susceptible bacteria. *Mycobacterium avium-intracellulare* is considerably more resistant than other mycobacteria (48).

Chlorhexidine is not sporicidal (discussed in "Mechanisms of resistance"). Even high concentrations of the bisbiguanide do not affect the viability of *Bacillus* spores at ambient temperatures (473, 474), although a marked sporicidal effect is achieved at elevated temperatures (475). Presumably, sufficient changes occur in the spore structure to permit an increased uptake of the biguanide, although this has yet to be shown experimentally. Little is known about the uptake of chlorhexidine by bacterial spores, although coatless forms take up more of the compound than do "normal" spores (474).

Chlorhexidine has little effect on the germination of bacterial spores (414, 422, 432, 447) but inhibits outgrowth (447). The reason for its lack of effect on the former process but its significant activity against the latter is unclear. It could, however, be reflected in the relative uptake of chlorhexidine, since germinating cells take up much less of the bisbiguanide than do outgrowing forms (474). Binding sites could thus be reduced in number or masked in germinating cells.

The antiviral activity of chlorhexidine is variable. Studies with different types of bacteriophages have shown that chlorhexidine has no effect on MS2 or K coliphages (300). High concentrations also failed to inactivate *Pseudomonas aeruginosa* phage F116 and had no effect on phage DNA within the capsid or on phage proteins (301); the transduction process was more sensitive to chlorhexidine and other biocides than was the phage itself. This substantiated an earlier finding (306) that chlorhexidine bound poorly to F116 particles. Chlorhexidine is not always considered a particularly effective antiviral agent, and its activity is restricted to the lipid-enveloped viruses (361). Chlorhexidine does not inactivate nonenveloped viruses such as rotavirus (485), HAV (315), or poliovirus (34). Its activity was found by Ranganathan (389) to be restricted to the nucleic acid core or the outer coat, although it is likely that the latter would be a more important target site.

Alexidine. Alexidine differs chemically from chlorhexidine in possessing ethylhexyl end groups. Alexidine is more rapidly bactericidal and produces a significantly faster alteration in bactericidal permeability (79, 80). Studies with mixed-lipid and pure phospholipid vesicles demonstrate that, unlike chlorhexidine, alexidine produces lipid phase separation and domain formation (Table 2). It has been proposed (80) that the nature of the ethylhexyl end group in alexidine, as opposed to the chlorophenol one in chlorhexidine, might influence the ability of a biguanide to produce lipid domains in the cytoplasmic membrane.

Polymeric biguanides. Vantocil is a heterodisperse mixture of polyhexamethylene biguanides (PHMB) with a molecular weight of approximately 3,000. Polymeric biguanides have found use as general disinfecting agents in the food industry and, very successfully, for the disinfection of swimming pools. Vantocil is active against gram-positive and gram-negative bacteria, although *P. aeruginosa* and *Proteus vulgaris* are less sensitive. Vantocil is not sporicidal. PHMB is a membrane-active agent that also impairs the integrity of the outer membrane of gram-negative bacteria, although the membrane may also act as a permeability barrier (64, 172). Activity of PHMB increases on a weight basis with increasing levels of polymerization, which has been linked to enhanced inner membrane perturbation (173, 174).

Unlike chlorhexidine but similar to alexidine (Table 2), PHMB causes domain formation of the acidic phospholipids of the cytoplasmic membrane (61–64, 172, 173, 227). Permeability changes ensue, and there is believed to be an altered function of some membrane-associated enzymes. The proposed sequence of events during its interaction with the cell envelope of *E. coli* is as follows: (i) there is rapid attraction of

PHMB toward the negatively charged bacterial cell surface, with strong and specific adsorption to phosphate-containing compounds; (ii) the integrity of the outer membrane is impaired, and PHMB is attracted to the inner membrane; (iii) binding of PHMB to phospholipids occurs, with an increase in inner membrane permeability (K^+ loss) accompanied by bacteriostasis; and (iv) complete loss of membrane function follows, with precipitation of intracellular constituents and a bactericidal effect.

Diamidines

The diamidines are characterized chemically as described in Table 1. The isethionate salts of two compounds, propamidine (4,4-diaminodiphenoxypropane) and dibromopropamidine (2,2-dibromo-4,4-diamidinodiphenoxypropane), have been used as antibacterial agents. Their antibacterial properties and uses were reviewed by Hugo (213) and Hugo and Russell (226). Clinically, diamidines are used for the topical treatment of wounds.

The exact mechanism of action of diamidines is unknown, but they have been shown to inhibit oxygen uptake and induce leakage of amino acids (Table 2), as would be expected if they are considered as cationic surface-active agents. Damage to the cell surface of *P. aeruginosa* and *Enterobacter cloacae* has been described (400).

Halogen-Releasing Agents

Chlorine- and iodine-based compounds are the most significant microbicidal halogens used in the clinic and have been traditionally used for both antiseptic and disinfectant purposes.

Chlorine-releasing agents. Excellent reviews that deal with the chemical, physical, and microbiological properties of chlorine-releasing agents (CRAs) are available (42, 130). The most important types of CRAs are sodium hypochlorite, chlorine dioxide, and the *N*-chloro compounds such as sodium dichloroisocyanurate (NaDCC), with chloramine-T being used to some extent. Sodium hypochlorite solutions are widely used for hard-surface disinfection (household bleach) and can be used for disinfecting spillages of blood containing human immunodeficiency virus or HBV. NaDCC can also be used for this purpose and has the advantages of providing a higher concentration of available chlorine and being less susceptible to inactivation by organic matter. In water, sodium hypochlorite ionizes to produce Na^+ and the hypochlorite ion, OCl^-, which establishes an equilibrium with hypochlorous acid, HOCl (42). Between pH 4 and 7, chlorine exists predominantly as HClO, the active moiety, whereas above pH9, OCl^- predominates. Although CRAs have been predominantly used as hard-surface disinfectants, novel acidified sodium chlorite (a two-component system of sodium chlorite and mandelic acid) has been described as an effective antiseptic (248).

Surprisingly, despite being widely studied, the actual mechanism of action of CRAs is not fully known (Table 2). CRAs are highly active oxidizing agents and thereby destroy the cellular activity of proteins (42); potentiation of oxidation may occur at low pH, where the activity of CRAs is maximal, although increased penetration of outer cell layers may be achieved with CRAs in the unionized state. Hypochlorous acid has long been considered the active moiety responsible for bacterial inactivation by CRAs, the OCl^- ion having a minute effect compared to undissolved HOCl (130). This correlates with the observation that CRA activity is greatest when the percentage of undissolved HOCl is highest. This concept applies to hypochlorites, NaDCC, and chloramine-T.

Deleterious effects of CRAs on bacterial DNA that involve the formation of chlorinated derivatives of nucleotide bases have been described (115, 128, 477). Hypochlorous acid has also been found to disrupt oxidative phosphorylation (26) and other membrane-associated activity (70). In a particularly interesting paper, McKenna and Davies (321) described the inhibition of bacterial growth by hypochlorous acid. At 50 μM (2.6 ppm), HOCl completely inhibited the growth of *E. coli* within 5 min, and DNA synthesis was inhibited by 96% but protein synthesis was inhibited by only 10 to 30%. Because concentrations below 5 mM (260 ppm) did not induce bacterial membrane disruption or extensive protein degradation, it was inferred that DNA synthesis was the sensitive target. In contrast, chlorine dioxide inhibited bacterial protein synthesis (33).

CRAs at higher concentrations are sporicidal (44, 421, 431); this depends on the pH and concentration of available chlorine (408, 412). During treatment, the spores lose refractivity, the spore coat separates from the cortex, and lysis occurs (268). In addition, a number of studies have concluded that CRA-treated spores exhibit increased permeability of the spore coat (131, 268, 412).

CRAs also possess virucidal activity (34, 46, 116, 315, 394, 407, 467, 485, 486). Olivieri et al. (359) showed that chlorine inactivated naked f2 RNA at the same rate as RNA in intact phage, whereas f2 capsid proteins could still adsorb to the host. Taylor and Butler (504) found that the RNA of poliovirus type 1 was degraded into fragments by chlorine but that poliovirus inactivation preceded any severe morphological changes. By contrast, Floyd et al. (149) and O'Brien and Newman (357) demonstrated that the capsid of poliovirus type 1 was broken down. Clearly, further studies are needed to explain the antiviral action of CRAs.

Iodine and iodophors. Although less reactive than chlorine, iodine is rapidly bactericidal, fungicidal, tuberculocidal, virucidal, and sporicidal (184). Although aqueous or alcoholic (tincture) solutions of iodine have been used for 150 years as antiseptics, they are associated with irritation and excessive staining. In addition, aqueous solutions are generally unstable; in solution, at least seven iodine species are present in a complex equilibrium, with molecular iodine (I_2) being primarily responsible for antimicrobial efficacy (184). These problems were overcome by the development of iodophors ("iodine carriers" or "iodine-releasing agents"); the most widely used are povidone-iodine and poloxamer-iodine in both antiseptics and disinfectants. Iodophors are complexes of iodine and a solubilizing agent or carrier, which acts as a reservoir of the active "free" iodine (184). Although germicidal activity is maintained, iodophors are considered less active against certain fungi and spores than are tinctures (454).

Similar to chlorine, the antimicrobial action of iodine is rapid, even at low concentrations, but the exact mode of action is unknown. Iodine rapidly penetrates into microorganisms (76) and attacks key groups of proteins (in particular the free-sulfur amino acids cysteine and methionine [184, 267]), nucleotides, and fatty acids (15, 184), which culminates in cell death (184). Less is known about the antiviral action of iodine, but nonlipid viruses and parvoviruses are less sensitive than lipid enveloped viruses (384). Similarly to bacteria, it is likely that iodine attacks the surface proteins of enveloped viruses, but they may also destabilize membrane fatty acids by reacting with unsaturated carbon bonds (486).

Silver Compounds

In one form or another, silver and its compounds have long been used as antimicrobial agents (55, 443). The most important silver compound currently in use is silver sulfadiazine

(AgSD), although silver metal, silver acetate, silver nitrate, and silver protein, all of which have antimicrobial properties, are listed in *Martindale, The Extra Pharmacopoeia* (312). In recent years, silver compounds have been used to prevent the infection of burns and some eye infections and to destroy warts.

Silver nitrate. The mechanism of the antimicrobial action of silver ions is closely related to their interaction with thiol (sulfydryl, –SH) groups (32, 49, 161, 164), although other target sites remain a possibility (397, 509). Liau et al (287) demonstrated that amino acids such as cysteine and other compounds such as sodium thioglycolate containing thiol groups neutralized the activity of silver nitrate against *P. aeruginosa*. By contrast, amino acids containing disulfide (SS) bonds, non-sulfur-containing amino acids, and sulfur-containing compounds such as cystathione, cysteic acid, L-methionine, taurine, sodium bisulfite, and sodium thiosulfate were all unable to neutralize Ag^+ activity. These and other findings imply that interaction of Ag^+ with thiol groups in enzymes and proteins plays an essential role in bacterial inactivation, although other cellular components may be involved. Hydrogen bonding, the effects of hydrogen bond-breaking agents, and the specificity of Ag^+ for thiol groups were discussed in greater detail by Russell and Hugo (443) (Table 2). Virucidal properties might also be explained by binding to –SH groups (510).

Lukens (292) proposed that silver salts and other heavy metals such as copper act by binding to key functional groups of fungal enzymes. Ag^+ causes the release of K^+ ions from microorganisms; the microbial plasma or cytoplasmic membrane, with which is associated many important enzymes, is an important target site for Ag^+ activity (161, 329, 392, 470).

In addition to its effects on enzymes, Ag^+ produces other changes in microorganisms. Silver nitrate causes marked inhibition of growth of *Cryptococcus neoformans* and is deposited in the vacuole and cell wall as granules (60). Ag^+ inhibits cell division and damages the cell envelope and contents of *P. aeruginosa* (398). Bacterial cells increase in size, and the cytoplasmic membrane, cytoplasmic contents, and outer cell layers all exhibit structural abnormalities, although without any blebs (protuberances) (398). Finally, the Ag^+ ion interacts with nucleic acids (543); it interacts preferentially with the bases in DNA rather than with the phosphate groups, although the significance of this in terms of its lethal action is unclear (231, 387, 510, 547).

Silver sulfadiazine. AgSD is essentially a combination of two antibacterial agents, Ag^+ and sulfadiazine (SD). The question whether the antibacterial effect of AgSD arises predominantly from only one of the compounds or via a synergistic interaction has been posed repeatedly. AgSD has a broad spectrum of activity and, unlike silver nitrate, produces surface and membrane blebs in susceptible (but not resistant) bacteria (96). AgSD binds to cell components, including DNA (332, 404). Based on a chemical analysis, Fox (153) proposed a polymeric structure of AgSD composed of six silver atoms bonding to six SD molecules by linkage of the silver atoms to the nitrogens of the SD pyrimidine ring. Bacterial inhibition would then presumably be achieved when silver binds to sufficient base pairs in the DNA helix, thereby inhibiting transcription. Similarly, its antiphage properties have been ascribed to the fact that AgSD binds to phage DNA (154, 388). Clearly, the precise mechanism of action of AgSD has yet to be solved.

Peroxygens

Hydrogen peroxide. Hydrogen peroxide (H_2O_2) is a widely used biocide for disinfection, sterilization, and antisepsis. It is a clear, colorless liquid that is commercially available in a variety of concentrations ranging from 3 to 90%. H_2O_2 is considered environmentally friendly, because it can rapidly degrade into the innocuous products water and oxygen. Although pure solutions are generally stable, most contain stabilizers to prevent decomposition. H_2O_2 demonstrates broad-spectrum efficacy against viruses, bacteria, yeasts, and bacterial spores (38). In general, greater activity is seen against gram-positive than gram-negative bacteria; however, the presence of catalase or other peroxidases in these organisms can increase tolerance in the presence of lower concentrations. Higher concentrations of H_2O_2 (10 to 30%) and longer contact times are required for sporicidal activity (416), although this activity is significantly increased in the gaseous phase. H_2O_2 acts as an oxidant by producing hydroxyl free radicals ($^{\bullet}OH$) which attack essential cell components, including lipids, proteins, and DNA. It has been proposed that exposed sulfhydryl groups and double bonds are particularly targeted (38).

Peracetic acid. Peracetic acid (PAA) (CH_3COOOH) is considered a more potent biocide than hydrogen peroxide, being sporicidal, bactericidal, virucidal, and fungicidal at low concentrations (<0.3%) (38). PAA also decomposes to safe by-products (acetic acid and oxygen) but has the added advantages of being free from decomposition by peroxidases, unlike H_2O_2, and remaining active in the presence of organic loads (283, 308). Its main application is as a low-temperature liquid sterilant for medical devices, flexible scopes, and hemodialyzers, but it is also used as an environmental surface sterilant (100, 308).

Similar to H_2O_2, PAA probably denatures proteins and enzymes and increases cell wall permeability by disrupting sulfhydryl (–SH) and sulfur (S–S) bonds (22, 38).

Phenols

Phenolic-type antimicrobial agents have long been used for their antiseptic, disinfectant, or preservative properties, depending on the compound. It has been known for many years (215) that, although they have often been referred to as "general protoplasmic poisons," they have membrane-active properties which also contribute to their overall activity (120) (Table 2).

Phenol induces progressive leakage of intracellular constituents, including the release of K^+, the first index of membrane damage (273), and of radioactivity from ^{14}C-labeled *E. coli* (242, 265). Pulvertaft and Lumb (386) demonstrated that low concentrations of phenols (0.032%, 320 μg/ml) and other (nonphenolic) agents lysed rapidly growing cultures of *E. coli*, staphylococci, and streptococci and concluded that autolytic enzymes were not involved. Srivastava and Thompson (487, 488) proposed that phenol acts only at the point of separation of pairs of daughter cells, with young bacterial cells being more sensitive than older cells to phenol.

Hugo and Bloomfield (216, 217) showed with the chlorinated bis-phenol fenticlor that there was a close relationship between bactericidal activity and leakage of 260-nm-absorbing material (leakage being induced only by bactericidal concentrations). Fentichlor affected the metabolic activities of *S. aureus* and *E. coli* (217) and produced a selective increase in permeability to protons with a consequent dissipation of the proton motive force (PMF) and an uncoupling of oxidative phosphorylation (41). Chlorocresol has a similar action (124). Coagulation of cytoplasmic constituents at higher phenol concentrations, which causes irreversible cellular damage, has been described by Hugo (215).

The phenolics possess antifungal and antiviral properties. Their antifungal action probably involves damage to the plas-

ma membrane (436), resulting in leakage of intracellular constituents. Phenol does not affect the transduction of *P. aeruginosa* PAO by bacteriophage F116 (301), has no effect on phage DNA within the capsid, and has little effect on several of the phage band proteins unless treatments of 20 min or longer are used (303, 304).

Bis-Phenols

The bis-phenols are hydroxy-halogenated derivatives of two phenolic groups connected by various bridges (191, 446). In general, they exhibit broad-spectrum efficacy but have little activity against *P. aeruginosa* and molds and are sporostatic toward bacterial spores. Triclosan and hexachlorophane are the most widely used biocides in this group, especially in antiseptic soaps and hand rinses. Both compounds have been shown to have cumulative and persistent effects on the skin (313).

Triclosan. Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether; Irgasan DP 300) exhibits particular activity against gram-positive bacteria (469, 521). Its efficacy against gram-negative bacteria and yeasts can be significantly enhanced by formulation effects. For example, triclosan in combination with EDTA caused increased permeability of the outer membrane (282). Reports have also suggested that in addition to its antibacterial properties, triclosan may have anti-inflammatory activity (25, 522). The specific mode of action of triclosan is unknown, but it has been suggested that the primary effects are on the cytoplasmic membrane. In studies with *E. coli*, triclosan at subinhibitory concentrations inhibited the uptake of essential nutrients, while higher, bactericidal concentrations resulted in the rapid release of cellular components and cell death (393). Studies with a divalent-ion-dependent *E. coli* triclosan mutant for which the triclosan MIC was 10-fold greater than that for a wild-type strain showed no significant differences in total envelope protein profiles but did show significant differences in envelope fatty acids (370). Specifically, a prominent 14:1 fatty acid was absent in the resistant strain, and there were minor differences in other fatty acid species. It was proposed that divalent ions and fatty acids may adsorb and limit the permeability of triclosan to its site of action (370). Minor changes in fatty acid profiles were recently found in both *E. coli* and *S. aureus* strains for which the triclosan MICs were elevated; however, the MBCs were not affected, suggesting, as for other phenols, that the cumulative effects on multiple targets contribute to the bactericidal activity (318, 319).

Hexachlorophene. Hexachlorophene (hexachlorophane; 2,2′-dihydroxy-3,5,6,3′,5′,6′-hexachlorodiphenylmethane) is another bis-phenol whose mode of action has been extensively studied. The primary action of hexachlorophene, based on studies with *Bacillus megatherium*, is to inhibit the membrane-bound part of the electron transport chain, and the other effects noted above are secondary ones that occur only at high concentrations (92, 158, 241, 481). It induces leakage, causes protoplast lysis, and inhibits respiration. The threshold concentration for the bactericidal activity of hexachlorphene is 10 μg/ml (dry weight), but peak leakage occurs at concentrations higher than 50 μg/ml and cytological changes occur above 30 μg/ml. Furthermore, hexachlorophene is bactericidal at 0°C despite causing little leakage at this temperature. Despite the broad-spectrum efficacy of hexachlorophene, concerns about toxicity (256), in particular in neonates, have meant that its use in antiseptic products has been limited.

Halophenols

Chloroxylenol (4-chloro-3,5-dimethylphenol; *p*-chloro-*m*-xylenol) is the key halophenol used in antiseptic or disinfectant formulations (66). Chloroxylenol is bactericidal, but *P. aeruginosa* and many molds are highly resistant (66, 432). Surprisingly, its mechanism of action has been little studied despite its widespread use over many years. Because of its phenolic nature, it would be expected to have an effect on microbial membranes.

Quaternary Ammonium Compounds

Surface-active agents (surfactants) have two regions in their molecular structures, one a hydrocarbon, water-repellent (hydrophobic) group and the other a water-attracting (hydrophilic or polar) group. Depending on the basis of the charge or absence of ionization of the hydrophilic group, surfactants are classified into cationic, anionic, nonionic, and ampholytic (amphoteric) compounds. Of these, the cationic agents, as exemplified by quaternary ammonium compounds (QACs), are the most useful antiseptics and disinfectants (160). They are sometimes known as cationic detergents. QACs have been used for a variety of clinical purposes (e.g., preoperative disinfection of unbroken skin, application to mucous membranes, and disinfection of noncritical surfaces). In addition to having antimicrobial properties, QACs are also excellent for hard-surface cleaning and deodorization.

It has been known for many years that QACs are membrane-active agents (221) (Table 2) (i.e., with a target site predominantly at the cytoplasmic (inner) membrane in bacteria or the plasma membrane in yeasts) (215). Salton (460) proposed the following sequence of events with microorganisms exposed to cationic agents: (i) adsorption and penetration of the agent into the cell wall; (ii) reaction with the cytoplasmic membrane (lipid or protein) followed by membrane disorganization; (iii) leakage of intracellular low-molecular-weight material; (iv) degradation of proteins and nucleic acids; and (v) wall lysis caused by autolytic enzymes. There is thus a loss of structural organization and integrity of the cytoplasmic membrane in bacteria, together with other damaging effects to the bacterial cell (120).

Useful information about the selectivity of membrane action can be obtained by studying the effects of biocides on protoplasts and spheroplasts suspended in various solutes. QACs cause lysis of spheroplasts and protoplasts suspended in sucrose (107, 215, 243, 428). The cationic agents react with phospholipid components in the cytoplasmic membrane (69), thereby producing membrane distortion and protoplast lysis under osmotic stress. Isolated membranes do not undergo disaggregation on exposure to QACs, because the membrane distortion is not sufficiently drastic. The non-QAC agents TCC and trichlorosalicylanide have specific effects: TCC induces protoplast lysis in ammonium chloride by increasing Cl^- permeability, whereas trichlorosalicylanide induces lysis in ammonium nitrate by increasing NO_3^- permeability (428). In contrast, QACs (and chlorhexidine) induce lysis of protoplasts or spheroplasts suspended in various solutes because they effect generalized, rather than specific, membrane damage.

The bacterial cytoplasmic membrane provides the mechanism whereby metabolism is linked to solute transport, flagellar movement, and the generation of ATP. Protons are extruded to the exterior of the bacterial cell during metabolism. The combined potential (concentration or osmotic effect of the proton and its electropositivity) is the PMF, which drives these ancillary activities (428). The QAC cetrimide was found (121) to have an effect on the PMF in *S. aureus*. At its bacteriostatic concentration, cetrimide caused the discharge of the pH component of the PMF and also produced the maximum amount of 260-nm-absorbing material.

QACs are also believed to damage the outer membrane of gram-negative bacteria, thereby promoting their own uptake. This aspect of QACs is considered below (see "Intrinsic resistance of gram-negative bacteria").

The QAC cetylpyridium chloride (CPC) induces the leakage of K^+ and pentose material from the yeast *S. cerevisiae* and induces protoplast lysis as well as interacting with crude cell sap (205). Unlike chlorhexidine, however, no biphasic effect on protoplast lysis was observed. The initial toxic effect of QACs on yeast cells is a disorganization of the plasma membranes, with organized lipid structures in the membranes (and in lipid bilayers) being disrupted.

QACs are sporostatic; they inhibit the outgrowth of spores (the development of a vegetative cell from a germinated spore) but not the actual germination processes (development from dormancy to a metabolically active state), albeit by an unknown mechanism (414). Likewise, the QACs are not mycobactericidal but have a mycobacteriostatic action, although the actual effects on mycobacteria have been little studied (419).

The QACs have an effect on lipid, enveloped (including human immunodeficiency virus and HBV) but not nonenveloped viruses (394, 485, 486). QAC-based products induced disintegration and morphological changes of human HBV, resulting in loss of infectivity (382). In studies with different phages (298–301, 303–305, 307), CPC significantly inhibited transduction by bacteriophage F116 and inactivated the phage particles. Furthermore, CPC altered the protein bands of F116 but did not affect the phage DNA within the capsid.

Vapor-Phase Sterilants

Many heat-sensitive medical devices and surgical supplies can be effectively sterilized by liquid sterilants (in particular glutaraldehyde, PAA, and hydrogen peroxide) or by vapor-phase sterilization systems (Table 1). The most widely used active agents in these "cold" systems are ethylene oxide, formaldehyde and, more recently developed, hydrogen peroxide and PAA. Ethylene oxide and formaldehyde are both broad-spectrum alkylating agents. However, their activity is dependent on active concentration, temperature, duration of exposure, and relative humidity (87). As alkylating agents, they attack proteins, nucleic acids, and other organic compounds; both are particularly reactive with sulfhydryl and other enzyme-reactive groups. Ethylene oxide gas has the disadvantages of being mutagenic and explosive but is not generally harsh on sensitive equipment, and toxic residuals from the sterilization procedure can be routinely eliminated by correct aeration. Formaldehyde gas is similar and has the added advantage of being nonexplosive but is not widely used in health care. Vapor-phase hydrogen peroxide and PAA are considered more active (as oxidants) at lower concentrations than in the liquid form (334). Both active agents are used in combination with gas plasma in low-temperature sterilization systems (314). Their main advantages over other vapor-phase systems include low toxicity, rapid action, and activity at lower temperature; the disadvantages include limited penetrability and applications.

MECHANISMS OF RESISTANCE

Introduction

As stated above, different types of microorganisms vary in their response to antiseptics and disinfectants. This is hardly surprising in view of their different cellular structure, composition, and physiology. Traditionally, microbial susceptibility to antiseptics and disinfectants has been classified based on these differences; with recent work, this classification can be further extended (Fig. 1). Because different types of organisms react differently, it is convenient to consider bacteria, fungi, viruses, protozoa, and prions separately.

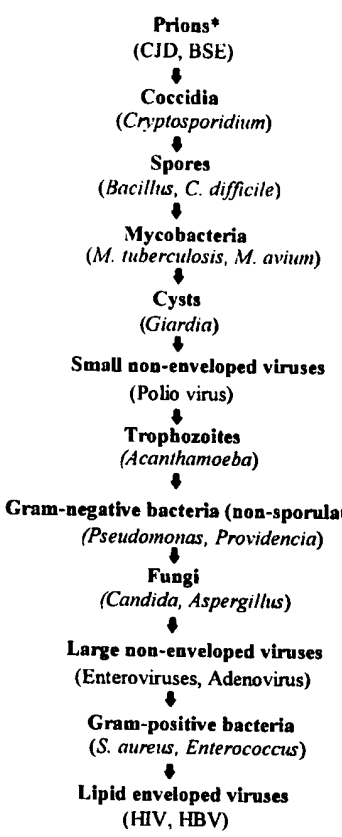


FIG. 1. Descending order of resistance to antiseptics and disinfectants. The asterisk indicates that the conclusions are not yet universally agreed upon.

Bacterial Resistance to Antiseptics and Disinfectants

In recent years, considerable progress has been made in understanding more fully the responses of different types of bacteria (mycobacteria, nonsporulating bacteria, and bacterial spores) to antibacterial agents (43, 84, 414, 415, 419, 422, 496). As a result, resistance can be either a natural property of an organism (intrinsic) or acquired by mutation or acquisition of plasmids (self-replicating, extrachromosomal DNA) or transposons (chromosomal or plasmid integrating, transmissible DNA cassettes). Intrinsic resistance is demonstrated by gram-negative bacteria, bacterial spores, mycobacteria, and, under certain conditions, staphylococci (Table 5). Acquired, plasmid-mediated resistance is most widely associated with mercury compounds and other metallic salts. In recent years, acquired resistance to certain other types of biocides has been observed, notably in staphylococci.

Intrinsic Bacterial Resistance Mechanisms

For an antiseptic or disinfectant molecule to reach its target site, the outer layers of a cell must be crossed. The nature and composition of these layers depend on the organism type and may act as a permeability barrier, in which there may be a reduced uptake (422, 428). Alternatively but less commonly, constitutively synthesized enzymes may bring about degradation of a compound (43, 214, 358). Intrinsic (innate) resistance

TABLE 5. Intrinsic resistance mechanisms in bacteria to antiseptics and disinfectants

Type of resistance	Example(s)	Mechanism of resistance
Impermeability		
Gram-negative bacteria	QACs, triclosan, diamines	Barrier presented by outer membrane may prevent uptake of antiseptic or disinfectant; glycocalyx may also be involved
Mycobacteria	Chlorhexidine, QACs Glutaraldehyde	Waxy cell wall prevents adequate biocide entry Reason for high resistance of some strains of *M. chelonae*(?)
Bacterial spores	Chlorhexidine, QACs, phenolics	Spore coat(s) and cortex present a barrier to entry of antiseptics and disinfectants
Gram-positive bacteria	Chlorhexidine	Glycocalyx/mucoexopolysaccaride may be associated with reduced diffusion of antiseptic
Inactivation (chromosomally mediated)	Chlorohexidine	Breakdown of chlorhexidine molecule may be responsible for resistance

is thus a natural, chromosomally controlled property of a bacterial cell that enables it to circumvent the action of an antiseptic or disinfectant. Gram-negative bacteria tend to be more resistant than gram-positive organisms, such as staphylococci (Table 6).

Intrinsic resistance of bacterial spores. Bacterial spores of the genera *Bacillus* and *Clostridium* have been widely studied and are invariably the most resistant of all types of bacteria to antiseptics and disinfectants (43, 46, 150, 414, 418, 420, 422, 423, 457). Although *Bacillus* species are generally not pathogenic, their spores are widely used as indicators of efficient sterilization. *Clostridium* species are significant pathogens; for example, *C. difficile* is the most common cause of hospital-acquired diarrhea (478). Many biocides are bactericidal or bacteristatic at low concentrations for nonsporulating bacteria, including the vegetative cells of *Bacillus* and *Clostridium* species, but high concentrations may be necessary to achieve a sporicidal effect (e.g., for glutaraldehyde and CRAs). By contrast, even high concentrations of alcohol, phenolics, QACs, and chlorhexidine lack a sporicidal effect, although this may be achieved when these compounds are used at elevated temperatures (475).

A typical spore has a complex structure (29, 151). In brief, the germ cell (protoplast or core) and germ cell wall are surrounded by the cortex, outside which are the inner and outer spore coats. A thin exosporium may be present in the spores of some species but may surround just one spore coat. RNA, DNA, and DPA, as well as most of the calcium, potassium, manganese, and phosphorus, are present in the spore protoplast. Also present are large amounts of low-molecular-weight basic proteins (small acid-soluble spore proteins [SASPs]), which are rapidly degraded during germination. The cortex consists largely of peptidoglycan, including a spore-specific muramic lactam. The spore coats comprise a major portion of the spore. These structures consist largely of protein, with an alkali-soluble fraction made up of acidic polypeptides being found in the inner coat and an alkali-resistant fraction associated with the presence of disulfide-rich bonds being found in the outer coat. These aspects, especially the roles of the coat(s) and cortex, are all relevant to the mechanism(s) of resistance presented by bacterial spores to antiseptics and disinfectants.

Several techniques are available for studying mechanisms of spore resistance (428). They include removing the spore coat and cortex by using a "step-down" technique to achieve a highly synchronous sporulation (so that cellular changes can be accurately monitored), employing spore mutants that do not sporulate beyond genetically determined stages in sporulation, adding an antiseptic or disinfectant at the commencement of sporulation and determining how far the process can proceed, and examining the role of SASPs. Such procedures have helped provide a considerable amount of useful information. Sporulation itself is a process in which a vegetative cell develops into a spore and involves seven stages (designated 0 to VII). During this process, the vegetative cell (stage 0) undergoes a series of morphological changes that culminate in the release of a mature spore (stage VII). Stages IV (cortex development) to VII are the most important in the development of resistance to biocides.

Resistance to antiseptics and disinfectants develops during sporulation and may be an early, intermediate, or (very) late event (103, 375, 378, 429, 474). Useful markers for monitoring the development of resistance are toluene (resistance to which is an early event), heat (intermediate), and lysozyme (late) (236, 237). Studies with a wild-type *B. subtilis* strain, 168, and its Spo$^-$ mutants have helped determine the stages at which resistance develops (262, 375, 474). From these studies (Fig. 2), the order of development of resistance was toluene (marker), formaldehyde, sodium lauryl sulfate, phenol, and phenylmercuric nitrate; *m*-cresol, chlorocresol, chlorhexidine gluconate, cetylpyridinium chloride, and mercuric chloride; and moist heat (marker), sodium dichloroisocyanurate, sodium hypochlorite, lysozyme (marker), and glutaraldehyde. The association of the onset of resistance to a particular antiseptic or disinfectant with a particular stage(s) in spore development is thereby demonstrated.

Spore coat-less forms, produced by treatment of spores un-

TABLE 6. MIC of some antiseptics and disinfectants against gram-positive and gram-negative bacteria[a]

Chemical agent	MIC (μg/ml) for:		
	S. aureus[b]	*E. coli*	*P. aeruginosa*
Benzalkonium chloride	0.5	50	250
Benzethonium chloride	0.5	32	250
Cetrimide	4	16	64–128
Chlorhexidine	0.5–1	1	5–60
Hexachlorophene	0.5	12.5	250
Phenol	2,000	2,000	2,000
o-Phenylphenol	100	500	1,000
Propamine isethionate	2	64	256
Dibromopropamidine isethionate	1	4	32
Triclosan	0.1	5	>300

[a] Based on references 226 and 440.
[b] MICs of cationic agents for some MRSA strains may be higher (see Table 10).

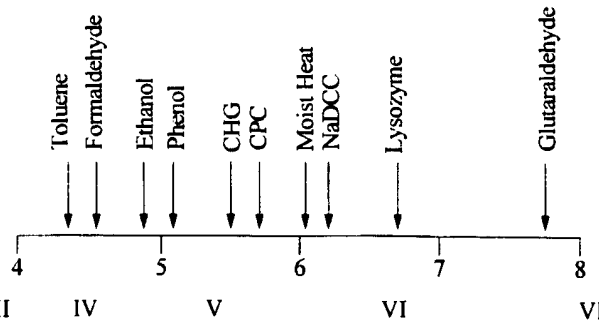


FIG. 2. Development of resistance of *Bacillus subtilis* during sporulation. Roman numerals indicate the sporulation stage from III (engulfment of the forespore) to VII (release of the mature spore). Arabic numbers indicate the time (hours) following the onset of sporulation and the approximate times at which resistance develops against biocides (262). CHG, chlorhexidine; CPC, cetylpyridinium chloride; NaDCC, sodium dichloroisocyanurate.

der alkaline conditions with urea plus dithiothreitol plus sodium lauryl sulfate (UDS), have also been of value in estimating the role of the coats in limiting the access of antiseptics and disinfectants to their target sites. However, Bloomfield and Arthur (44, 45) and Bloomfield (43) showed that this treatment also removes a certain amount of cortex and that the amount of cortex remaining can be further reduced by the subsequent use of lysozyme. These findings demonstrate that the spore coats have an undoubted role in conferring resistance but that the cortex also is an important barrier since (UDS plus lysozyme)-treated spores are much more sensitive to chlorine- and iodine-releasing agents than are UDS-exposed spores.

The initial development and maturity of the cortex are implicated in the development of resistance to phenolics. Likewise, it is now clear that cortex development is at least partially responsible for resistance to chlorhexidine and QACs; this resistance is enhanced in developing spores by the initiation of spore coat synthesis (262). The effect of various concentrations of chlorhexidine, sublethal to vegetative bacteria, on the development of spores of *B. subtilis* 168 MB_2 were investigated by Knott and Russell (261). They found that chlorhexidine affected spore development; as concentrations of the biguanide increased, spore index values (the percentage of cells forming spores) decreased and sensitivity to both heat and toluene increased. By contrast, the control (untreated) culture was highly resistant to both of these agents and had a high spore index value, indicative of high levels of mature spores. The slightly increased resistance to toluene compared to resistance to heat was not surprising, since cells must reach stages V to VI (synthesis of spore coats and maturation) to attain heat resistance but only stage III (forespore engulfment) to attain toluene resistance (Fig. 2); in other words, if sporulation is inhibited by chlorhexidine, more cells are likely to reach stage III than the later stages. While less definitive than the earlier approaches, these procedures provide further evidence of the involvement of the cortex and coats in chlorhexidine resistance.

Development of resistance during sporulation to formaldehyde was an early event but depended to some extent on the concentration (1 to 5% [vol/vol]) of formaldehyde used. This appears to be at odds with the extremely late development of resistance to the dialdehyde, glutaraldehyde. Since glutaraldehyde and the monoaldehyde, formaldehyde, contain an aldehyde group(s) and are alkylating agents, it would be plausible to assume that they would have a similar mode of sporicidal action, even though the dialdehyde is a more powerful alkylating agent. If this were true, it could also be assumed that spores would exhibit the same resistance mechanisms for these disinfectants. In aqueous solution, formaldehyde forms a glycol in equilibrium (512, 524); thus, formaldehyde could well be acting poorly as an alcohol-type disinfectant rather than as an aldehyde (327). Alkaline glutaraldehyde does not readily form glycols in aqueous solution (178). Resistance to formaldehyde may be linked to cortex maturation, and resistance to glutaraldehyde may be linked to coat formation (262).

Setlow and his coworkers (472) demonstrated that α/β-type SASPs coat the DNA in wild-type spores of *B. subtilis*, thereby protecting it from attack by enzymes and antimicrobial agents. Spores ($\alpha^-\beta^-$) lacking these α/β-type SASPs are significantly more sensitive to hydrogen peroxide (471) and hypochlorite (456). Thus, SASPs contribute to spore resistance to peroxide and hypochlorite but may not be the only factors involved, since the coats and cortex also play a role (428).

Two other aspects of spores should be considered: the revival of injured spores and the effects of antiseptics and disinfectants on germinating and outgrowing spores. Although neither aspect is truly a resistance mechanism, each can provide useful information about the site and mechanism of action of sporicidal agents and about the associated spore resistance mechanisms and might be of clinical importance.

The revival of disinfectant-treated spores has not been extensively studied. Spicher and Peters (483, 484) demonstrated that formaldehyde-exposed spores of *B. subtilis* could be revived after a subsequent heat shock process. A more recent finding with *B. stearothermophilus* casts further doubt on the efficacy of low-temperature steam with formaldehyde as a sterilizing procedure (541). The revival of spores exposed to glutaraldehyde, formaldehyde, chlorine, and iodine was examined by Russell and his colleagues (103, 376, 377, 424, 532–537). A small proportion of glutaraldehyde-treated spores of various *Bacillus* species were revived when the spores were treated with alkali after neutralization of glutaraldehyde with glycine (103, 379, 380). Experiments designed to distinguish between germination and outgrowth in the revival process have demonstrated that sodium hydroxide-induced revival increases the potential for germination. Based on other findings, the germination process is also implicated in the revival of spores exposed to other disinfectants.

Intrinsic resistance of mycobacteria. Mycobacteria are well known to possess a resistance to antiseptics and disinfectants that is roughly intermediate between those of other nonsporulating bacteria and bacterial spores (Fig. 1) (177, 345, 419). There is no evidence that enzymatic degradation of harmful molecules takes place. The most likely mechanism for the high resistance of mycobacteria is associated with their complex cell walls that provide an effective barrier to the entry of these agents. To date, plasmid- or transposon-mediated resistance to biocides has not been demonstrated in mycobacteria.

The mycobacterial cell wall is a highly hydrophobic structure with a mycoylarabinogalactan-peptidoglycan skeleton (27, 105, 106, 322, 389, 390, 461, 530). The peptidoglycan is covalently linked to the polysaccharide copolymer (arabinogalactan) made up of arabinose and galactose esterified to mycolic acids. Also present are complex lipids, lipopolysaccharides (LPSs), and proteins, including porin channels through which hydrophilic molecules can diffuse into the cell (232, 356). Similar cell wall structures exist in all the mycobacterial species examined to date (228). The cell wall composition of a particular species may be influenced by its environmental niche (27). Pathogenic bacteria such as *Mycobacterium tuberculosis* exist in a relatively nutrient-rich environment, whereas saprophytic mycobacteria living in soil or water are exposed to natural antibiotics and tend to be more intrinsically resistant to these drugs.

Antiseptics or disinfectants that exhibit mycobacterial activity are phenol, PAA, hydrogen peroxide, alcohol, and glutaraldehyde (16, 17, 99, 419, 425, 455). By contrast, other well-known bactericidal agents, such as chlorhexidine and QACs, are mycobacteristatic even when used at high concentrations (51, 52, 419, 425, 455). However, the activity of these can be substantially increased by formulation effects. Thus, a number of QAC-based products claim to have mycobacterial activity. For example, a newer formulation (Sactimed-I-Sinald) containing a mixture of alkyl polyguanides and alkyl QACs is claimed to be mycobactericidal (211, 353). However, there is some doubt whether the antibacterial agents had been properly quenched or neutralized to prevent carryover of inhibitory concentrations into recovery media.

Many years ago, it was proposed (T. H. Shen, cited in reference 99) that the resistance of mycobacteria to QACs was related to the lipid content of the cell wall. In support of this contention, *Mycobacterium phlei*, which has a low total cell lipid content, was more sensitive than *M. tuberculosis*, which has a higher lipid content. It was also noted that the resistance of various species of mycobacteria was related to the content of waxy material in the wall. It is now known that because of the highly hydrophobic nature of the cell wall, hydrophilic biocides are generally unable to penetrate the mycobacterial cell wall in sufficiently high concentrations to produce a lethal effect. However, low concentrations of antiseptics and disinfectants such as chlorhexidine must presumably traverse this permeability barrier, because the MICs are of the same order as those concentrations inhibiting the growth of nonmycobacterial strains such as *S. aureus*, although *M. avium-intracellulare* may be particularly resistant (51, 52). The component(s) of the mycobacterial cell wall responsible for the high biocide resistance are currently unknown, although some information is available. Inhibitors of cell wall synthesis increase the susceptibility of *M. avium* to drugs (391); inhibition of myocide C, arabinogalactan, and mycolic acid biosynthesis enhances drug susceptibility. Treatment of this organism with *m*-fluoro-DL-phenylalanine (*m*-FL-phe), which inhibits mycocide C synthesis, produces significant alterations in the outer cell wall layers (106). Ethambutol, an inhibitor of arabinogalactan (391, 501) and phospholipid (461, 462) synthesis, also disorganizes these layers. In addition, ethambutol induces the formation of ghosts without the dissolution of peptidoglycan (391). Methyl-4-(2-octadecylcyclopropen-1-yl) butanoate (MOCB) is a structural analogue of a key precursor in mycolic acid synthesis. Thus, the effects of MOCB on mycolic acid synthesis and *m*-FL-phe and ethambutol on outer wall biosynthetic processes leading to changes in cell wall architecture appear to be responsible for increasing the intracellular concentration of chemotherapeutic drugs. These findings support the concept of the cell wall acting as a permeability barrier to these drugs (425). Fewer studies have been made of the mechanisms involved in the resistance of mycobacteria to antiseptics and disinfectants. However, the activity of chlorhexidine and of a QAC, cetylpyridinium chloride, against *M. avium* and *M. tuberculosis* can be potentiated in the presence of ethambutol (52). From these data, it may be inferred that arabinogalactan is one cell wall component that acts as a permeability barrier to chlorhexidine and QACs. It is not possible, at present, to comment on other components, since these have yet to be investigated. It would be useful to have information about the uptake into the cells of these antiseptic agents in the presence and absence of different cell wall synthesis inhibitors.

One species of mycobacteria currently causing concern is *M. chelonae*, since these organisms are sometimes isolated from endoscope washes and dialysis water. One such strain was not killed even after a 60-min exposure to alkaline glutaraldehyde; in contrast, a reference strain showed a 5-log-unit reduction after a contact time of 10 min (519). This glutaraldehyde-resistant *M. chelonae* strain demonstrated an increased tolerance to PAA but not to NaDCC or to a phenolic. Other workers have also observed an above-average resistance of *M. chelonae* to glutaraldehyde and formaldehyde (72) but not to PAA (187, 294). The reasons for this high glutaraldehyde resistance are unknown. However, *M. chelonae* is known to adhere strongly to smooth surfaces, which may render cells within a biofilm less susceptible to disinfectants. There is no evidence to date that uptake of glutaraldehyde by *M. chelonae* is reduced.

Intrinsic resistance of other gram-positive bacteria. The cell wall of staphylococci is composed essentially of peptidoglycan and teichoic acid. Neither of these appears to act as an effective barrier to the entry of antiseptics and disinfectants. Since high-molecular-weight substances can readily traverse the cell wall of staphylococci and vegetative *Bacillus* spp., this may explain the sensitivity of these organisms to many antibacterial agents including QACs and chlorhexidine (411, 417, 422, 428, 451).

However, the plasticity of the bacterial cell envelope is a well-known phenomenon (381). Growth rate and any growth-limiting nutrient will affect the physiological state of the cells. Under such circumstances, the thickness and degree of cross-linking of peptidoglycan are likely to be modified and hence the cellular sensitivity to antiseptics and disinfectants will be altered. For example, Gilbert and Brown (171) demonstrated that the sensitivity of *Bacillus megaterium* cells to chlorhexidine and 2-phenoxyethanol is altered when changes in growth rate and nutrient limitation are made with chemostat-grown cells. However, lysozyme-induced protoplasts of these cells remained sensitive to, and were lysed by, these membrane-active agents. Therefore, the cell wall in whole cells is responsible for their modified response.

In nature, *S. aureus* may exist as mucoid strains, with the cells surrounded by a slime layer. Nonmucoid strains are killed more rapidly than mucoid strains by chloroxylenol, cetrimide, and chlorhexidine, but there is little difference in killing by phenols or chlorinated phenols (263); removal of slime by washing rendered the cells sensitive. Therefore, the slime plays a protective role, either as a physical barrier to disinfectant penetration or as a loose layer interacting with or absorbing the biocide molecules.

There is no evidence to date that vancomycin-resistant enterococci or enterococci with high-level resistance to aminoglycoside antibiotics are more resistant to disinfectants than are antibiotic-sensitive enterococcal strains (9, 11, 48, 319). However, enterococci are generally less sensitive to biocides than are staphylococci, and differences in inhibitory and bactericidal concentrations have also been found among enterococcal species (257).

Intrinsic resistance of gram-negative bacteria. Gram-negative bacteria are generally more resistant to antiseptics and disinfectants than are nonsporulating, nonmycobacterial gram-positive bacteria (Fig. 2) (428, 440, 441). Examples of MICs against gram-positive and -negative organisms are provided in Table 6. Based on these data, there is a marked difference in the sensitivity of *S. aureus* and *E. coli* to QACs (benzalkonium, benzethonium, and cetrimide), hexachlorophene, diamidines, and triclosan but little difference in chlorhexidine susceptibility. *P. aeruginosa* is considerably more resistant to most of these agents, including chlorhexidine, and (not shown) *Proteus* spp. possess an above-average resistance to cationic agents such as chlorhexidine and QACs (311, 440).

The outer membrane of gram-negative bacteria acts as a barrier that limits the entry of many chemically unrelated types

TABLE 7. Possible transport of some antiseptics and disinfectants into gram-negative bacteria[a]

Antiseptic/disinfectant	Passage across OM[b]	Passage across IM[b]
Chlorhexidine	Self-promoted uptake(?)	IM is a major target site; damage to IM enables biocide to enter cytosol, where further interaction occurs
QACs	Self-promoted uptake(?); also, OM might present a barrier	IM is a major target site; damage to IM enables biocide to enter cytosol, where further interaction occurs
Phenolics	Hydrophobic pathway (activity increases as hydrophobicity of phenolic increases)	IM is a major target site, but high phenolic concentrations coagulate cytoplasmic constituents

[a] Data from references 197, 433 to 435, 438, and 439.
[b] OM, outer membrane; IM, inner membrane.

of antibacterial agents (18, 169, 196, 197, 355, 366, 440, 516, 517). This conclusion is based on the relative sensitivities of staphylococci and gram-negative bacteria and also on studies with outer membrane mutants of *E. coli*, *S. typhimurium*, and *P. aeruginosa* (134, 135, 433–435, 438). Smooth, wild-type bacteria have a hydrophobic cell surface; by contrast, because of the phospholipid patches on the cell surface, deep rough (heptose-less) mutants are hydrophobic. These mutants tend to be hypersensitive to hydrophobic antibiotics and disinfectants. Low-molecular-weight (M_r <ca. 600) hydrophilic molecules readily pass via the porins into gram-negative cells, but hydrophobic molecules diffuse across the outer membrane bilayer (Table 7). In wild-type gram-negative bacteria, intact LPS molecules prevent ready access of hydrophobic molecules to phospholipid and thence to the cell interior. In deep rough strains, which lack the O-specific side chain and most of the core polysaccharide, the phospholipid patches at the cell surface have their head groups oriented toward the exterior.

In addition to these hydrophilic and hydrophobic entry pathways, a third pathway has been proposed for cationic agents such as QACs, biguanidies, and diamidines. It is claimed that these damage the outer membrane, thereby promoting their own uptake (197). Polycations disorganize the outer membrane of *E. coli* (520). It must be added, however, that the QACs and diamidines are considerably less active against wild-type strains than against deep rough strains whereas chlorhexidine has the same order of activity (MIC increase about 2 to 3 fold) against both types of *E. coli* strains (434, 435, 439). However, *S. typhimurium* mutants are more sensitive to chlorhexidine than are wild-type strains (433).

Gram-negative bacteria that show a high level of resistance to many antiseptics and disinfectants include *P. aeruginosa*, *Burkholderia cepacia*, *Proteus* spp., and *Providencia stuartii* (428, 440). The outer membrane of *P. aeruginosa* is responsible for its high resistance; in comparison with other organisms, there are differences in LPS composition and in the cation content of the outer membrane (54). The high Mg^{2+} content aids in producing strong LPS-LPS links; furthermore, because of their small size, the porins may not permit general diffusion through them. *B. cepacia* is often considerably more resistant in the hospital environment than in artificial culture media (360); the high content of phosphate-linked arabinose in its LPS decreases the affinity of the outer membrane for polymyxin antibiotics and other cationic and polycationic molecules (97, 516). *Pseudomonas stutzeri*, by contrast, is highly sensitive to many antibiotics and disinfectants (449), which implies that such agents have little difficulty in crossing the outer layers of the cells of this organism.

Members of the genus *Proteus* are invariably insensitive to chlorhexidine (311). Some strains that are highly resistant to chlorhexidine, QACs, EDTA, and diamidines have been isolated from clinical sources. The presence of a less acidic type of outer membrane LPS could be a contributing factor to this intrinsic resistance (97, 516).

A particularly troublesome member of the genus *Providencia* is *P. stuartii*. Like *Proteus* spp., *P. stuartii* strains have been isolated from urinary tract infections in paraplegic patients and are resistant to different types of antiseptics and disinfectants including chlorhexidine and QACs (492, 496). Strains of *P. stuartii* that showed low-, intermediate-, and high-level resistance to chlorhexidine formed the basis of a series of studies of the resistance mechanism(s) (86, 422, 428). Gross differences in the composition of the outer layers of these strains were not detected, and it was concluded that (i) subtle changes in the structural arrangement of the cell envelopes of these strains was associated with this resistance and (ii) the inner membrane was not implicated (230).

Few authors have considered peptidoglycan in gram-negative bacteria as being a potential barrier to the entry of inhibitory substances. The peptidoglycan content of these organisms is much lower than in staphylococci, which are inherently more sensitive to many antiseptics and disinfectants. Nevertheless, there have been instances (discussed in reference 422) where gram-negative organisms grown in subinhibitory concentrations of a penicillin have deficient permeability barriers. Furthermore, it has been known for many years (215, 409, 411) that penicillin-induced spheroplasts and lysozyme-EDTA-Tris "protoplasts" of gram-negative bacteria are rapidly lysed by membrane-active agents such as chlorhexidine. It is conceivable that the stretched nature of both the outer and inner membranes in β-lactam-treated organisms could contribute to this increased susceptibility.

The possibility exists that the cytoplasmic (inner) membrane provides one mechanism of intrinsic resistance. This membrane is composed of lipoprotein and would be expected to prevent passive diffusion of hydrophilic molecules. It is also known that changes in membrane composition affect sensitivity to ethanol (159). Lannigan and Bryan (275) proposed that decreased susceptibility of *Serratia marcescens* to chlorhexidine was linked to the inner membrane, but Ismaeel et al. (230) could find no such role with chlorhexidine-resistant *P. stuartii*. At present, there is little evidence to implicate the inner membrane in biocide resistance. In addition, chlorhexidine degradation was reported for *S. marcescens*, *P. aeruginosa*, and *Achromobacter/Alcaligenes xylosoxidans* (358).

Physiological (phenotypic) adaption as an intrinsic mechanism. The association of microorganisms with solid surfaces leads to the generation of a biofilm, defined as a consortium of organisms organized within an extensive exopolysaccharide exopolymer (93, 94). Biofilms can consist of monocultures, of several diverse species, or of mixed phenotypes of a given species (57, 73, 381). Some excellent publications that deal with the nature, formation, and content of biofilms are available (125, 178, 276, 538). Biofilms are important for several reasons.

TABLE 8. Biofilms and microbial response to antimicrobial agents

Mechanism of resistance associated with biofilms	Comment
Exclusion or reduced access of antiseptic or disinfectant to underlying cell	Depends on (i) nature of antiseptic/disinfectant, (ii) binding capacity of glycocalyx toward antiseptic or disinfectant, and (iii) rate of growth of microcolony relative to diffusion rate of chemical inhibitor.
Modulation of microenvironment	Associated with (i) nutrient limitation and (ii) growth rate
Increased production of degradative enzymes by attached cells	Mechanism unclear at present
Plasmid transfer between cells in biofilm?	Associated with enhanced tolerance to antiseptics and disinfectants?

notably biocorrosion, reduced water quality, and foci for contamination of hygienic products (10, 12–14). Colonization also occurs on implanted biomaterials and medical devices, resulting in increased infection rates and possible recurrence of infection (125).

Bacteria in different parts of a biofilm experience different nutrient environments, and their physiological properties are affected (57). Within the depths of a biofilm, for example, nutrient limitation is likely to reduce growth rates, which can affect susceptibility to antimicrobial agents (98, 142, 171, 172). Thus, the phenotypes of sessile organisms within biofilms differ considerably from the planktonic cells found in laboratory cultures (73). Slow-growing bacteria are particularly insusceptible, a point reiterated recently in another context (126).

Several reasons can account for the reduced sensitivity of bacteria within a biofilm (Table 8). There may be (i) reduced access of a disinfectant (or antibiotic) to the cells within the biofilm, (ii) chemical interaction between the disinfectant and the biofilm itself, (iii) modulation of the microenvironment, (iv) production of degradative enzymes (and neutralizing chemicals), or (v) genetic exchange between cells in a biofilm. However, bacteria removed from a biofilm and recultured in culture media are generally no more resistant than the "ordinary" planktonic cells of that species (57).

Several instances are known of the contamination of antiseptic or disinfectant solutions by bacteria. For example, Marrie and Costerton (310) described the prolonged survival of *S. marcescens* in 2% chlorhexidine solutions, which was attributed to the embedding of these organisms in a thick matrix that adhered to the walls of a storage containers. Similar conclusions were reached by Hugo et al. (225) concerning the survival of *B. cepacia* in chlorhexidine and by Anderson et al. (10, 12–14) concerning the contamination of iodophor antiseptics with *Pseudomonas*. In the studies by Anderson et al., *Pseudomonas* biofilms were found on the interior surfaces of polyvinyl chloride pipes used during the manufacture of providone-iodine antiseptics. It is to be wondered whether a similar reason could be put forward for the contamination by *S. marcescens* of a benzalkonium chloride solution implicated in meningitis (468). Recently, a novel strategy was described (540) for controlling biofilms through generation of hydrogen peroxide at the biofilm-surface interface rather than simply applying a disinfectant extrinsically. In this procedure, the colonized surface incorporated a catalyst that generated the active compound from a treatment agent.

Gram-negative pathogens can grow as biofilms in the catheterized bladder and are able to survive concentrations of chlorhexidine that are effective against organisms in noncatheterized individuals (493, 494). Interestingly, the permeability agent EDTA has only a temporary potentiating effect in the catheterized bladder, with bacterial growth subsequently recurring (495). *B. cepacia* freshly isolated from the hospital environment is often considerably more resistant to chlorhexidine than when grown in artificial culture media, and a glycocalyx may be associated with intrinsic resistance to the bisbiguanide (360). *Legionella pneumophila* is often found in hospital water distribution systems and cooling towers. Chlorination in combination with continuous heating (60°C) of incoming water is usually the most important disinfection measure; however, because of biofilm production, contaminating organisms may be less susceptible to this treatment (140). Increased resistance to chlorine has been reported for *Vibrio cholerae*, which expresses an amorphous exopolysaccharide causing cell aggregation ("rugose" morphology [336]) without any loss in pathogenicity.

One can reach certain conclusions about biofilms. The interaction of bacteria with surfaces is usually reversible and eventually irreversible. Irreversible adhesion is initiated by the binding of bacteria to the surface through exopolysaccharide glycocalyx polymers. Sister cells then arise by cell division and are bound within the glycocalyx matrix. The development of adherent microcolonies is thereby initiated, so that eventually a continuous biofilm is produced on the colonized surface. Bacteria within these biofilms reside in specific microenvironments that differ from those of cells grown under normal laboratory conditions and thus show variations in their response to antiseptics and disinfectants.

Recent nosocomial outbreaks due to *M. chelonae* (discussed under "Intrinsic resistance of mycobacteria"), *M. tuberculosis* (4, 323) and HCV (53) underscore the importance of pseudobiofilm formation in flexible fiberoptic scope contamination. These outbreaks were associated with inadequate cleaning of scopes, which compromised subsequent sterilization with glutaraldehyde. While these organisms do not form a true biofilm, the cross-linking action of glutaraldehyde can cause a buildup of insoluble residues and associated microorganisms on scopes and in automated reprocessors.

Biofilms provide the most important example of how physiological (phenotypic) adaptation can play a role in conferring intrinsic resistance (57). Other examples are also known. For example, fattened cells of *S. aureus* produced by repeated subculturing in glycerol-containing media are more resistant to alkyl phenols and benzylpenicillin than are wild-type strains (220). Subculture of these cells in routine culture media resulted in reversion to sensitivity (218). Planktonic cultures grown under conditions of nutrient limitation or reduced growth rates have cells with altered sensitivity to disinfectants, probably as a consequence of modifications in their outer membranes (56, 59, 98). In addition, many aerobic microorganisms have developed intrinsic defense systems that confer tolerance to peroxide stress (in particular H_2O_2) in vivo. The so-called oxidative-stress or SOS response has been well studied in *E. coli* and *Salmonella* and includes the production of neutralizing enzymes to prevent cellular damage (including peroxidases, catalases, glutathione reductase) and to repair DNA lesions (e.g., exonuclease III) (112, 114, 497). In both organisms, increased tolerance can be obtained by pretreatment with a subinhibitory dose of hydrogen peroxide (113, 539). Pretreatment induces a series of proteins, many of which are under the positive control of a sensor/regulator protein (OxyR), including catalase and glutathione reductase (497)

TABLE 9. Possible mechanisms of plasmid-encoded resistance to antiseptics and disinfectants

Chemical agent	Examples	Mechanisms
Antiseptics or disinfectants	Chlorhexidine salts	(i) Inactivation: not yet found to be plasmid mediated; chromosomally mediated inactivation; (ii) efflux: some *S. aureus*, some *S. epidermidis*; (iii) Decreased uptake(?)
	QACs	(i) Efflux: some *S. aureus*, some *S. epidermidis*; (ii) Decreased uptake(?)
	Silver compounds	Decreased uptake; no inactivation (cf. mercury compounds)
	Formaldehyde	(i) Inactivation by formaldehyde dehydrogenase; (ii) Cell surface alterations (outer membrane proteins)
	Acridines[a]	Efflux: some *S. aureus*, some *S. epidermidis*
	Diamidines	Efflux: some *S. aureus*, some *S. epidermidis*
	Crystal violet[a]	Efflux: some *S. aureus*, some *S. epidermidis*
Other biocides	Mercurials[b]	Inactivation (reductases, lyases)
	Ethidium bromide	Efflux: some *S. aureus*, some *S. epidermidis*

[a] Now rarely used for antiseptic or disinfectant purposes.
[b] Organomercurials are still used as preservatives.

and further nonessential proteins that accumulate to protect the cell (338). Cross-resistance to heat, ethanol, and hypochlorous acid has also been reported (81, 128, 335). The oxidative stress response in gram-positive bacteria is less well studied, but *Bacillus* tolerance to H_2O_2 has been described to vary during the growth phase (127) and in mutant strains (67, 200). Similar inducible defense mechanisms were described for *Campylobacter jejuni* (185), *Deinococcus* (528), and *Haemophilus influenzae* (36). However, the level of increased tolerance to H_2O_2 during the oxidative stress response may not afford significant protection to concentrations used in antiseptics and disinfectants (generally >3%). For example, *B. subtilis* mutants have been described to be more resistant at ~0.5% H_2O_2 than are wild-type strains at ~0.34% H_2O_2 (200).

Acquired Bacterial Resistance Mechanisms

As with antibiotics and other chemotherapeutic drugs, acquired resistance to antiseptics and disinfectants can arise by either mutation or the acquisition of genetic material in the form of plasmids or transposons. It is important to note that "resistance" as a term can often be used loosely and in many cases must be interpreted with some prudence. This is particularly true with MIC analysis. Unlike antibiotics, "resistance," or an increase in the MIC of a biocide, does not necessarily correlate with therapeutic failure. An increase in an antibiotic MIC can may have significant consequences, often indicating that the target organism is unaffected by its antimicrobial action. Increased biocide MICs due to acquired mechanisms have also been reported and in some case misinterpreted as indicating resistance. It is important that issues including the pleiotropic action of most biocides, bactericidal activity, concentrations used in products, direct product application, formulation effects, etc., be considered in evaluating the clinical implications of these reports.

Plasmids and bacterial resistance to antiseptics and disinfectants. Chopra (82, 83) examined the role of plasmids in encoding resistance (or increased tolerance) to antiseptics and disinfectants; this topic was considered further by Russell (413). It was concluded that apart from certain specific examples such as silver, other metals, and organomercurials, plasmids were not normally responsible for the elevated levels of antiseptic or disinfectant resistance associated with certain species or strains. Since then, however, there have been numerous reports linking the presence of plasmids in bacteria with increased tolerance to chlorhexidine, QACs, and triclosan, as well as to diamidines, acridines and ethidium bromide, and the topic was reconsidered (83, 423, 427) (Table 9).

Plasmid-encoded resistance to antiseptics and disinfectants had at one time been most extensively investigated with mercurials (both inorganic and organic), silver compounds, and other cations and anions. Mercurials are no longer used as disinfectants, but phenylmercuric salts and thiomersal are still used as preservatives in some types of pharmaceutical products (226). Resistance to mercury is plasmid borne, inducible, and may be transferred by conjugation or transduction. Inorganic mercury (Hg^{2+}) and organomercury resistance is a common property of clinical isolates of *S. aureus* containing penicillinase plasmids (110). Plasmids conferring resistance to mercurials are either narrow spectrum, specifying resistance to Hg^{2+} and to some organomercurials, or broad-spectrum, with resistance to the above compounds and to additional organomercurials (331). Silver salts are still used as topical antimicrobial agents (50, 443). Plasmid-encoded resistance to silver has been found in *Pseudomonas stutzeri* (192), members of the *Enterobacteriaceae* (479, 480, 511), and *Citrobacter* spp. (511). The mechanism of resistance has yet to be elucidated fully but may be associated with silver accumulation (152, 511).

(i) Plasmid-mediated antiseptic and disinfectant resistance in gram-negative bacteria. Occasional reports have examined the possible role of plasmids in the resistance of gram-negative bacteria to antiseptics and disinfectants. Sutton and Jacoby (498) observed that plasmid RP1 did not significantly alter the resistance of *P. aeruginosa* to QACs, chlorhexidine, iodine, or chlorinated phenols, although increased resistance to hexachlorophene was observed. This compound has a much greater effect on gram-positive than gram-negative bacteria, so that it is difficult to assess the significance of this finding. Transformation of this plasmid (which encodes resistance to carbenicillin, tetracycline, and neomycin and kanamycin) into *E. coli* or *P. aeruginosa* did not increase the sensitivity of these organisms to a range of antiseptics (5).

Strains of *Providencia stuartii* may be highly tolerant to Hg^{2+}, cationic disinfectants (such as chlorhexidine and QACs), and antibiotics (496). No evidence has been presented to show that there is a plasmid-linked association between antibiotic resistance and disinfectant resistance in these organisms, pseudomonads, or *Proteus* spp. (492). High levels of disinfectant resistance have been reported in other hospital isolates (195), although no clear-cut role for plasmid-specified resistance has emerged (102, 250, 348, 373, 518). High levels of tolerance to chlorhexidine and QACs (311) may be intrinsic or may have resulted from mutation. It has been proposed (492, 496) that the extensive usage of these cationic agents could be responsible for the selection of antiseptic-disinfectant-, and antibiotic-resistant strains; however, there is little evidence to support this conclusion. All of these studies demonstrated that it was difficult to transfer chlorhexidine or QAC resistance under nor-

TABLE 10. *qac* genes and susceptibility of *S. aureus* strains to some antiseptics and disinfectants

qac gene[a]	MIC ratios[b] of[c]:							
	Proflavine	CHG	Pt	Pi	CTAB	BZK	CPC	DC
qacA	>16	2.5	>16	>16	4	>3	>4	2
qacB	8	1	>4	2	2	>3	>2	2
qacC	1	1	ca. 1	1	6	>3	>4	1
qacD	1	1	ca. 1	1	6	>3	>4	1
MIC (μg/ml) for sensitive strain	40	0.8	<50	50[d]	1	<2	<1	4

[a] *qac* genes are otherwise known as nucleic acid binding (NAB) compound resistance genes (88).

[b] Calculated from the data in reference 289. Ratios are MICs for strains of *S. aureus* carrying various *qac* genes divided by the MIC for a strain carrying no gene (the actual MIC for the test strain is shown in the bottom row).

[c] CHG, chlorhexidine diacetate; Pt, pentamidine isethionate; Pi, propamidime isethionate; CTAB, cetyltrimethylammonium bromide; BZK, benzalkonium chloride; CPC, cetylpyridinium chloride; DC, dequalinium chloride.

[d] The MIC of propamidine isethionate for the sensitive *S. aureus* is considerably higher than the normal quoted value (ca. 2 μg/ml [Table 6]).

mal conditions and that plasmid-mediated resistance to these chemicals in gram-negative bacteria was an unlikely event. By contrast, plasmid R124 alters the OmpF outer membrane protein in *E. coli*, and cells containing this plasmid are more resistant to a QAC (cetrimide) and to other agents (406).

Bacterial resistance mechanisms to formaldehyde and industrial biocides may be plasmid encoded (71, 193). Alterations in the cell surface (outer membrane proteins [19, 246]) and formaldehyde dehydrogenase (247, 269) are considered to be responsible (202). In addition, the so-called TOM plasmid encodes enzymes for toluene and phenol degradation in *B. cepacia* (476).

(ii) Plasmid-mediated antiseptic and disinfectant resistance in staphylococci. Methicillin-resistant *S. aureus* (MRSA) strains are a major cause of sepsis in hospitals throughout the world, although not all strains have increased virulence. Many can be referred to as "epidemic" MRSA because of the ease with which they can spread (91, 295, 317). Patients at particularly high risk are those who are debilitated or immunocompromised or who have open sores.

It has been known for several years that some antiseptics and disinfectants are, on the basis of MICs, somewhat less inhibitory to *S. aureus* strains that contain a plasmid carrying genes encoding resistance to the aminoglycoside antibiotic gentamicin (Table 10). These biocidal agents include chlorhexidine, diamidines, and QACs, together with ethidium bromide and acridines (8, 238, 289, 368, 423, 427, 463). According to Mycock (346), MRSA strains showed a remarkable increase in tolerance (at least 5,000-fold) to povidone-iodine. However, there was no decrease in susceptibility of antibiotic-resistant strains to phenolics (phenol, cresol, and chlorocresol) or to the preservatives known as parabens (8).

Tennent et al. (505) proposed that increased resistances to cetyltrimethylammonium bromide (CTAB) and to propamidine isethionate were linked and that these cationic agents may be acting as a selective pressure for the retention of plasmids encoding resistance to them. The potential clinical significance of this finding remains to be determined.

Staphylococci are the only bacteria in which the genetic aspects of plasmid-mediated antiseptic and disinfectant resistant mechanisms have been described (466). In *S. aureus*, these mechanisms are encoded by at least three separate multidrug resistance determinants (Tables 10 and 11). Increased antiseptic MICs have been reported to be widespread among MRSA strains and to be specified by two gene families (*qacAB* and *qacCD*) of determinants (188, 280, 281, 288, 289, 363–365, 367, 506). The *qacAB* family of genes (Table 11) encodes proton-dependant export proteins that develop significant homology to other energy-dependent transporters such as the tetracycline transporters found in various strains of tetracycline-resistant bacteria (405). The *qacA* gene is present predominantly on the pSK1 family of multiresistance plasmids but is also likely to be present on the chromosome of clinical *S. aureus* strains as an integrated family plasmid or part thereof. The *qacB* gene is detected on large heavy-metal resistance plasmids. The *qacC* and *qacD* genes encode identical phenotypes and show restriction site homology; the *qacC* gene may have evolved from *qacD* (288).

Interesting studies by Reverdy et al. (395, 396), Dussau et al. (129) and Behr et al. (31) demonstrated a relationship between increased *S. aureus* MICs to the β-lactam oxacillin and four antiseptics (chlorhexidine, benzalkonium chloride, hexamine, and acriflavine). A gene encoding multidrug resistance was not found in susceptible strains but was present in 70% of *S. aureus* strains for which the MICs of all four of these antiseptics were increased and in 45% of the remaining strains resistant to at least one of these antiseptics (31). Genes encoding increased QAC tolerance may be widespread in food-associated staphylococcal species (203). Some 40% of isolates of coagulase-negative staphylococci (CNS) contain both *qacA* and *qacC* genes, with a possible selective advantage in possessing both as opposed to *qacA* only (281). Furthermore, there is growing evidence that *S. aureus* and CNS have a common pool of resistance determinants.

Triclosan is used in surgical scrubs, soaps, and deodorants. It is active against staphylococci and less active against most gram-negative organisms, especially *P. aeruginosa*, probably by virtue of a permeability barrier (428). Low-level transferable resistance to triclosan was reported in MRSA strains (88, 90); however, no further work on these organisms has been described. According to Sasatsu et al. (465), the MICs of triclosan against sensitive and resistant *S. aureus* strains were 100 and

TABLE 11. *qac* genes and resistance to quaternary ammonium compounds and other antiseptics and disinfectants

Multidrug resistance determinant[a]	Gene location	Resistance encoded to
qacA	pSK1 family of multiresistant plasmids, also β-lactamase and heavy-metal resistance families	QACs, chlorhexidine salts, diamidines, acridines, ethidium bromide
qacB	β-Lactamase and heavy-metal resistance plasmids	QACs, acridines, ethidium bromide
qacC[b]	Small plasmids (<3 kb) or large conjugative plasmids	Some QACs, ethidium bromide
qacD[b]	Large (50-kb) conjugative, multiresistance plasmids	Some QACs, ethidium bromide

[a] The *qacK* gene has also been described, but it is likely to be less significant than *qacAB* in terms of antiseptic or disinfectant tolerance.

[b] These genes have identical target sites and show restriction site homology.

>6,400 μg/ml, respectively; these results were disputed because these concentrations are well in excess of the solubility of triclosan (515), which is practically insoluble in water. Sasatsu et al. (464) described a high-level resistant strain of *S. aureus* for which the MICs of chlorhexidine, CTAB, and butylparaben were the same as for a low-level resistant strain. Furthermore, the MIC quoted for methylparaben comfortably exceeds its aqueous solubility. Most of these studies with sensitive and "resistant" strains involved the use of MIC evaluations (for example, Table 6). A few investigations examined the bactericidal effects of antiseptics. Cookson et al. (89) pointed out that curing of resistance plasmids produced a fall in MICs but not a consistent decrease in the lethal activity of chlorhexidine. There is a poor correlation between MIC and the rate of bactericidal action of chlorhexidine (88, 89, 319) and triclosan (90, 319). McDonnell et al. (318, 319) have described methicillin-susceptible *S. aureus* (MSSA) and MRSA strains with increased triclosan MICs (up to 1.6 μg/ml) but showed that the MBCs for these strains were identical; these results were not surprising, considering that biocides (unlike antibiotics) have multiple cellular targets. Irizarry et al. (229) compared the susceptibility of MRSA and MSSA strains to CPC and chlorhexidine by both MIC and bactericidal testing methods. However, the conclusion of this study that MRSA strains were more resistant warrants additional comments. On the basis of rather high actual MICs, MRSA strains were some four times more resistant to chlorhexidine and five times more resistant to a QAC (CPC) than were MSSA strains. At a concentration in broth of 2 μg of CPC/ml, two MRSA strains grew normally with a threefold increase in viable numbers over a 4-h test period whereas an MSSA strain showed a 97% decrease in viability. From this, the authors concluded that it was reasonable to speculate that the residual amounts of antiseptics and disinfectants found in the hospital environment could contribute to the selection and maintenance of multiresistant MRSA strains. Irizarry et al. (229) also concluded that MRSA strains are less susceptible than MSSA strains to both chronic and acute exposures to antiseptics and disinfectants. However, their results with 4 μg of CPC/ml show no such pattern: at this higher concentration, the turbidities (and viability) of the two MRSA and one MSSA strains decreased at very similar rates (if anything, one MRSA strain appeared to be affected to a slightly greater extent that the MSSA strain). Furthermore, the authors stated that chlorhexidine gave similar results to CPC. It is therefore difficult to see how Irizarry et al. arrived at their highly selective conclusions.

Plasmid-mediated efflux pumps are particularly important mechanisms of resistance to many antibiotics (85), metals (349), and cationic disinfectants and antiseptics such as QACs, chlorhexidine, diamidines, and acridines, as well as to ethidium bromide (239, 289, 324–336, 363–368). Recombinant *S. aureus* plasmids transferred into *E. coli* are responsible for conferring increased MICs of cationic agents to the gram-negative organism (505, 544). Midgley (324, 325) demonstrated that a plasmid-borne, ethidium resistance determinant from *S. aureus* cloned in *E. coli* encodes resistance to ethidium bromide and to QACs, which are expelled from the cells. A similar efflux system is present in *Enterococcus hirae* (326).

Sasatsu et al. (463) showed that duplication of *ebr* is responsible for resistance to ethidium bromide and to some antiseptics. Later, Sasatsu et al. (466) examined the origin of *ebr* (now known to be identical to *qacCD*) in *S. aureus*; *ebr* was found in antibiotic-resistant and -sensitive strains of *S. aureus*, CNS, and enterococcal strains. The nucleotide sequences of the amplified DNA fragment from sensitive and resistant strains were identical, and it was proposed that in antiseptic-resistant cells there was an increase in the copy number of the *ebr* (*qacCD*) gene whose normal function was to remove toxic substances from normal cells of staphylococci and enterococci.

Based on DNA homology, it was proposed that *qacA* and related genes carrying resistance determinants evolved from preexisting genes responsible for normal cellular transport systems (405) and that the antiseptic resistance genes evolved before the introduction and use of topical antimicrobial products and other antiseptics and disinfectants (288, 289, 365, 367, 368, 405).

Baquero et al. (23) have pointed out that for antibiotics, the presence of a specific resistance mechanism frequently contributes to the long-term selection of resistant variants under in vivo conditions. Whether low-level resistance to cationic antiseptics, e.g., chlorhexidine, QACs, can likewise provide a selective advantage on staphylococci carrying *qac* genes remains to be elucidated. The evidence is currently contentious and inconclusive.

(iii) Plasmid-mediated antiseptic and disinfectant resistance in other gram-positive bacteria. Antibiotic-resistant corynebacteria may be implicated in human infections, especially in the immunocompromised. 'Group JK' coryneforms (*Corynebacterium jeikeium*) were found to be more tolerant than other coryneforms to the cationic disinfectants ethidium bromide and hexachlorophene, but studies with plasmid-containing and plasmid-cured derivatives produced no evidence of plasmid-associated resistance (285). *Enterococcus faecium* strains showing high level resistance to vancomycin, gentamicin, or both are not more resistant to chlorhexidine or other nonantibiotic agents (9, 11, 20, 319). Furthermore, despite the extensive dental use of chlorhexidine, strains of *Streptococcus mutans* remain sensitive to it (235). To date, therefore, there is little or no evidence of plasmid-associated resistance of nonstaphylococcal gram-positive bacteria to antiseptics and disinfectants.

Mutational resistance to antiseptics and disinfectants. Chromosomal mutation to antibiotics has been recognized for decades. By contrast, fewer studies have been performed to determine whether mutation confers resistance to antiseptics and disinfectants. It was, however, demonstrated over 40 years ago (77, 78) that *S. marcescens*, normally inhibited by QACs at <100 μg/ml, could adapt to grow in 100,000 μg of a QAC per ml. The resistant and sensitive cells had different surface characteristics (electrophoretic mobilities), but resistance could be lost when the cells were grown on QAC-free media. One problem associated with QACs and chlorhexidine is the turbidity produced in liquid culture media above a certain concentration (interaction with agar also occurs), which could undoubtedly interfere with the determination of growth. This observation is reinforced by the findings presented by Nicoletti et al. (354).

Prince et al. (383) reported that resistance to chlorhexidine could be induced in some organisms but not in others. For example, *P. mirabilis* and *S. marcescens* displayed 128- and 258-fold increases, respectively, in resistance to chlorhexidine, whereas it was not possible to develop resistance to chlorhexidine in *Salmonella enteritidis*. The resistant strains did not show altered biochemical properties of changed virulence for mice, and some strains were resistant to the QAC benzalkonium chloride. Moreover, resistance to chlorhexidine was stable in *S. marcescens* but not in *P. mirabilis*. Despite extensive experimentation with a variety of procedures, Fitzgerald et al. (148) were unable to develop stable chlorhexidine resistance in *E. coli* or *S. aureus*. Similar observations were made by Cookson et al. (89), who worked with MRSA and other strains of *S. aureus*, and by McDonnell et al. (319), who worked with MRSA and enterococci. Recently, stable chlorhexidine resistance was developed in *P. stutzeri* (502); these strains showed

various levels of increased tolerance to QACs, triclosan, and some antibiotics, probably as a result of a nonspecific alteration of the cell envelope (452). The adaptation and growth of *S. marcescens* in contact lens disinfectants containing chlorhexidine, with cross-resistance to a QAC, have been described previously (166).

Chloroxylenol-resistant strains of *P. aeruginosa* were isolated by repeated exposure in media containing gradually increasing concentrations of the phenolic, but the resistance was unstable (432). The adaptation of *P. aeruginosa* to QACs is a well-known phenomenon (1, 2, 240). Resistance to amphoteric surfactants has also been observed, and, interestingly, cross-resistance to chlorhexidine has been noted (240). This implies that the mechanism of such resistance is nonspecific and that it involves cellular changes that modify the response of organisms to unrelated biocidal agents. Outer membrane modification is an obvious factor and has indeed been found with QAC-resistant and amphoteric compound-resistant *P. aeruginosa* (240) and with chlorhexidine-resistant *S. marcescens* (166). Such changes involve fatty acid profiles and, perhaps more importantly, outer membrane proteins. It is also pertinent to note here the recent findings of Langsrud and Sundheim (274). In this study, resistance of *P. fluorescens* to QACs was reduced when EDTA was present with the QAC (although the lethal effect was mitigated after the cells were grown in medium containing QAC and EDTA); similar results have been found with laboratory-generated *E. coli* mutants for which the MICs of triclosan were increased (318). EDTA has long been known (175, 410) to produce changes in the outer membrane of gram-negative bacteria, especially pseudomonads. Thus, it appears that, again, the development of resistance is associated with changes in the cell envelope, thereby limiting uptake of antiseptics and disinfectants.

Hospital (as for other environmental) isolates of gram-negative bacteria are invariably less sensitive to disinfectants than are laboratory strains (196, 209, 279, 286, 492). Since plasmid-mediated transfer has apparently been ruled out (see above), selection and mutation could play an important role in the presence of these isolates. Subinhibitory antibiotic concentrations may cause subtle changes in the bacterial outer structure, thereby stimulating cell-to-cell contact (109); it remains to be tested if residual concentrations of antiseptics or disinfectants in clinical situations could produce the same effect.

Another insusceptibility mechanism has been put forward, in this instance to explain acridine resistance. It has been proposed (270, 351) that proflavine-sensitive and -resistant cells are equally permeable to the acridine but that resistant cells possessed the ability to expel the bound dye. This is an important point and one that has been reinforced by more recent studies that demonstrate the significance of efflux in resistance of bacteria to antibiotics (284, 330, 355). Furthermore, multidrug resistance (MDR) is a serious problem in enteric and other gram-negative bacteria. MDR is a term used to describe resistance mechanisms used by genes that form part of the normal cell genome (168). These genes are activated by induction or mutation caused by some types of stress, and because they are distributed ubiquitously, genetic transfer is not needed. Although such systems are most important in the context of antibiotic resistance, George (168) provides several examples of MDR systems in which an operon or gene is associated with changes in antiseptic or disinfectant susceptibility; e.g., (i) mutations at an *acr* locus in the Acr system render *E. coli* more sensitive to hydrophobic antibiotics, dyes, and detergents; (ii) the *robA* gene is responsible for overexpression in *E. coli* of the RobA protein that confers multiple antibiotic and heavy-metal resistance (interestingly, Ag^+ may be effluxed [350]); and (iii) the MarA protein controls a set of genes (*mar* and *soxRS* regulons) that confer resistance not only to several antibiotics but also to superoxide-generating agents. Moken et al. (333) have found that low concentrations of pine oil (used as a disinfectant) could select for *E. coli* mutants that overexpressed MarA and demonstrated low levels of cross-resistance to antibiotics. Deletion of the *mar* or *acrAB* locus (the latter encodes a PMF-dependant efflux pump) increased the susceptibility of *E. coli* to pine oil; deletion of *acrAB*, but not of *mar*, increased the susceptibility of *E. coli* to chloroxylenol and to a QAC. In addition, the *E. coli* MdfA (multidrug transporter) protein was recently identified and confers greater tolerance to both antibiotics and a QAC (benzalkonium) (132). The significance of these and other MDR systems in bacterial susceptibility to antiseptics and disinfectants, in particular the issue of cross-resistance with antibiotics, must be studied further. At present, it is difficult to translate these laboratory findings to actual clinical use, and some studies have demonstrated that antibiotic-resistant bacteria are not significantly more resistant to the lethal (or bactericidal) effects of antiseptic and disinfectants than are antibiotic-sensitive strains (11, 88, 89, 319).

TABLE 12. Possible mechanisms of fungal resistance to antiseptics and disinfectants

Type of resistance	Possible mechanism	Example(s)
Intrinsic	Exclusion	Chlorhexidine
	Enzymatic inactivation	Formaldehyde
	Phenotypic modulation	Ethanol
	Efflux	Not demonstrated to date[a]
Acquired	Mutation	Some preservative
	Inducible efflux	Some preservatives[a]
	Plasmid-mediated responses	Not demonstrated to date

[a] Efflux is now known to be one mechanism of fungal resistance to antibiotics (531).

Mechanisms of Fungal Resistance to Antiseptics and Disinfectants

In comparison with bacteria, very little is known about the ways in which fungi can circumvent the action of antiseptics and disinfectants (104, 111, 296). There are two general mechanisms of resistance (Table 12): (i) intrinsic resistance, a natural property or development of an organism (201); and (ii) acquired resistance. In one form of intrinsic resistance, the cell wall presents a barrier to reduce or exclude the entry of an antimicrobial agent. The evidence to date is somewhat patchy, but the available information links cell wall glucan, wall thickness, and relatively porosity to the susceptibility of *Saccharomyces cerevisiae* to chlorhexidine (Table 13) (204–208). Protoplasts of this organism prepared by glucuronidase in the presence of β-mercaptoethanol are lysed by chlorhexidine concentrations well below those effective against "normal" (whole) cells. Furthermore, culture age influences the response of *S. cerevisiae* to chlorhexidine; the cells walls are much less sensitive at stationary phase than at logarithmic growth phase (208), taking up much less [^{14}C]chlorhexidine gluconate (206). Gale (165) demonstrated a phenotypic increase in the resistance of *Candida albicans* to the polyenic antibiotic amphotericin B as the organisms entered the stationary growth phase, which was attributed to cell wall changes involving tighter cross-linking (74). Additionally, any factor increasing glucanase activity increased amphotericin sensitivity.

The porosity of the yeast cell wall is affected by its chemical

TABLE 13. Parameters affecting the response of *S. cerevisiae* to chlorhexidine[a]

Parameter	Role in susceptibility of cells to chlorhexidine
Cell wall composition	
Mannan	No role found to date
Glucan	Possible significance: at concentrations below those active against whole cells, chlorhexidine lyses protoplasts
Cell wall thickness	Increases in cells of older cultures: reduced chlorhexidine uptake responsible for decreased activity(?)
Relative porosity	Decreases in cells of older cultures: reduced chlorhexidine uptake responsible for decreased activity(?)
Plasma membrane	Changes altering CHG susceptibility(?); not investigated to date

[a] Data from references 204 to 208 and 436.

composition, with the wall acting as a barrier or modulator to the entry and exit of various agents. DeNobel et al. (117–119) used the uptake of fluorescein isothiocyanurate (FITC) dextrans and the periplasmic enzyme invertase as indicators of yeast cell wall porosity. Intact *S. cerevisiae* cells were able to endocytose FITC dextrans of 70 but not of 150. A new assay for determining the relative cell wall porosity in yeast based upon polycation-induced leakage of UV-absorbing compounds was subsequently developed. Hiom et al. (206, 208) found that the relative porosity of cells decreases with increasing culture age and that there was a reduced uptake of radiolabeled chlorhexidine gluconate. As the age of an *S. cerevisiae* culture increases, there is a significant increase in the cell wall thickness, with values of 0.19, 0.25, and 0.31 μm recorded for cells from 1-, 2-, and 6-day old cultures, respectively (206).

These findings (Table 13) can provide a tentative picture of the cellular factors that modify the response of *S. cerevisiae* to chlorhexidine. Mannan mutants of *S. cerevisiae* show a similar degree of sensitivity to chlorhexidine as the parent strain (204). The glucan layer is shielded from β-glucuronidase by mannoproteins, but this effect is overcome by β-mercaptoethanol (119). The mannoprotein consists of two fractions, sodium dodecyl sulfate-soluble mannoproteins and sodium dodecyl sulfate-insoluble, glucanase-soluble ones: the latter limit cell wall porosity (119). Thus, glucan (and possibly mannoproteins) plays a key role in determining the uptake and hence the activity of chlorhexidine in *S. cerevisiae*. *C. albicans* is less sensitive and takes up less [^{14}C]chlorhexidine overall (206), but only a few studies with this organism and with molds have been performed.

Yeasts grown under different conditions have variable levels of sensitivity to ethanol (176, 402). Cells with linoleic acid-enriched plasma membranes are more resistant to ethanol than are cells with oleic acid-enriched ones, from which it has been inferred that a more fluid membrane enhances ethanol resistance (6).

There is no evidence to date of antiseptic efflux (although benzoic acid in energized cells is believed to be eliminated by flowing down the electrochemical gradient [529]) and no evidence of acquired resistance by mutation (except to some preservatives [436]) or by plasmid-mediated mechanisms (426, 436). It is disappointing that so few rigorous studies have been performed with yeasts and molds and antiseptics and disinfectants (see also Miller's [328] treatise on mechanisms for reaching the site of action). Molds are generally more resistant than yeasts (Table 14) and considerably more resistant than nonsporulating bacteria (Table 15). Mold spores, although more resistant than nonsporulating bacteria, are less resistant than bacterial spores to antiseptics and disinfectants (436). It is tempting to speculate that the cell wall composition in molds confers a high level of intrinsic resistance on these organisms.

Mechanisms of Viral Resistance to Antiseptics and Disinfectants

Early studies on the effects of disinfectants on viruses were reviewed by Grossgebauer (189). Potential viral targets are the viral envelope, which contains lipids and is a typical unit membrane; the capsid, which is principally protein in nature; and the genome. An important hypothesis was put forward in 1963 (258) and modified in 1983 (259) in which it was proposed that viral susceptibility to disinfectants could be based on whether viruses were "lipophilic" in nature, because they possessed a lipid envelope (e.g., herpes simplex virus [259]) or "hydrophilic" because they did not (e.g., poliovirus [514]). Lipid-enveloped viruses were sensitive to lipophilic-type disinfectants, such as 2-phenylphenol, cationic surfactants (QACs), chlorhexidine, and isopropanol, as well as to ether and chloroform. Klein and Deforest (259) further classified viruses into three groups (Table 16), A (lipid containing), B (nonlipid picornaviruses), and C (other nonlipid viruses larger than those in group B) and disinfectants into two groups, broad-spectrum ones that inactivated all viruses and lipophilic ones that failed to inactivate picornoviruses and parvoviruses.

Capsid proteins are predominantly protein in nature, and biocides such as glutaraldehyde, hypochlorite, ethylene oxide, and hydrogen peroxide, which react strongly with amino or sulfhydryl groups might possess virucidal activity. It must, however, be added that destruction of the viral capsid may result in the release of a potentially infectious nucleic acid and that viral inactivation would only be complete if the viral nucleic acid is also destroyed.

Unfortunately, the penetration of antiseptics and disinfectants into different types of viruses and their interaction with viral components have been little studied, although some information has been provided by investigations with bacteriophages (307). Bacteriophages are being considered as "indicator species" for assessing the virucidal activity of disinfectants (108) and could thus play an increasing important role in this context; for example, repeated exposure of *E. coli* phage f2 to chlorine was claimed to increase its resistance to disinfection (542).

Thurman and Gerber (509, 510) pointed out that conflicting results on the actions of disinfectants on different virus types were often reported, and they suggested that the structural integrity of a virus was altered by an agent that reacted with viral capsids to increase viral permeability. Thus, a "two-stage"

TABLE 14. Lethal concentrations of antiseptics and disinfectants toward some yeasts and molds[a]

Antimicrobial agent[b]	Lethal concn (μg/μl) toward:		
	Yeast (*Candida albicans*)	Molds	
		Penicillium chrysogenum	*Aspergillus niger*
QACs			
Benzalkonium chloride	10	100–200	100–200
Cetrimide/CTAB	25	100	250
Chlorhexidine	20–40	400	200

[a] Derived in part from data in reference 525.
[b] CTAB, cetyltrimethylammonium bromide.

TABLE 15. Kinetic approach: *D*-values at 20°C of phenol and benzalkonium chloride against fungi and bacteria[a]

Antimicrobial agent	pH	Concn (%, wt/vol)	*D*-value (h)[b] against:				
			Aspergillus niger	*Candida albicans*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Phenol	5.1	0.5	20	13.5	0.94	—[c]	0.66
	6.1	0.5	32.4	18.9	1.72	0.17	1.9
Benzalkonium chloride	5.1	0.001	—[d]	9.66	0.06	3.01	3.12
	6.1	0.002	—[d]	5.5	—[c]	0.05	0.67

[a] Abstracted from the data in references 244 and 245.
[b] *D*-values are the times to reduce the viable population by 1 log unit.
[c] Inactivation was so rapid that the *D*-values could not be measured.
[d] No inactivation; fungistatic effect only.

disinfection system could be an efficient means of viral inactivation while overcoming the possibility of multiplicity reactivation (first put forward by Luria [293]) to explain an initial reduction and then an increase in the titer of disinfectant-treated bacteriophage. Multiplicity reactivation as a mechanism of resistance was supported by the observation of Young and Sharp (546) that clumping of poliovirus following partial inactivation by hypochlorite significantly increased the phage titer. It is envisaged as consisting of random damage to the capsid protein or nucleic acid of clumped, noninfectious virions from which complementary reconstruction of an infectious particle occurs by hybridization with the gene pool of the inactivated virions (298).

Another resistance mechanism also involves viral aggregation, e.g., the persistence of infectivity of formaldehyde-treated poliovirus (458) and the resistance of Norwalk virus to chlorination (249). A typical biphasic survival curve of enterovirus and rotavirus exposed to peracetic acid is also indicative of the presence of viral aggregates (198).

Finally, there remains the possibility of viral adaptation to new environmental conditions. In this context, Bates et al. (28) described the development of poliovirus having increased resistance to chlorine inactivation. Clearly, much remains to be learned about the mechanism of viral inactivation by and viral resistance to disinfectants.

Mechanisms of Protozoal Resistance to Antiseptics and Disinfectants

Intestinal protozoa, such as *Cryptosporidium parvum*, *Entamoeba histolytica*, and *Giardia intestinalis*, are all potentially pathogenic to humans and have a resistant, transmissible cyst (or oocyst for *Cryptosporidium*) (233, 234). Of the disinfectants available currently, ozone is the most effective protozoan cysticide, followed by chlorine dioxide, iodine, and free chlorine, all of which are more effective than the chloramines (234, 264). Cyst forms are invariably the most resistant to chemical disinfectants (Fig. 1). The reasons for this are unknown, but it would be reasonable to assume that cysts, similar to spores, take up fewer disinfectant molecules from solution than do vegetative forms.

Some recent studies have compared the responses of cysts and trophozoites of *Acanthamoeba castellanii* to disinfectants used in contact lens solutions and monitored the development of resistance during encystation and the loss of resistance during excystation (251–255). The lethal effects of chlorhexidine and of a polymeric biguanide were time and concentration dependent, and mature cysts were more resistant than preencystment trophozoites or preexcystment cysts. The cyst "wall" appeared to act as a barrier to the uptake of these agents, thereby presenting a classical type of intrinsic resistance mechanism (163). Acanthamoebae are capable of forming biofilms on surfaces such as contact lenses (186). Although protozoal biofilms have yet to be studied extensively in terms of their response to disinfectants, it is apparent that they could play a significant role in modulating the effects of chemical agents.

Mechanisms of Prion Resistance to Disinfectants

The transmissible degenerative encephalopathies (TDEs) form a group of fatal neurological diseases of humans and other animals. TDEs are caused by prions, abnormal proteinaceous agents that appear to contain no agent-specific nucleic acid (385). An abnormal protease-resistant form (PrP^{res}) of a normal host protein is implicated in the pathological process.

Prions are considered highly resistant to physical and chemical agents (Fig. 1), although the fact that crude preparations are often studied means that extraneous materials could, at least to some extent, mask the true efficacy of these agents (503). According to Taylor (503), there is currently no known decontamination procedure that will guarantee the complete absence of infectivity in TDE-infected tissues processed by histopathological procedures. Prions survive acid treatment, but a synergistic effect with autoclaving plus sodium hydroxide treatment is observed. Formaldehyde, unbuffered glutaraldehyde (acidic pH), and ethylene oxide have little effect on infectivity, although chlorine-releasing agents (especially hypochlorites), sodium hydroxide, some phenols, and guanidine thiocyanate are more effective (141, 309, 503).

With the information presently available, it is difficult to explain the extremely high resistance of prions, save to comment that the protease-resistant protein is abnormally stable to degradative processes.

CONCLUSIONS

It is clear that microorganisms can adapt to a variety of environmental physical and chemical conditions, and it is therefore not surprising that resistance to extensively used antiseptics and disinfectants has been reported. Of the mechanisms that have been studied, the most significant are clearly intrinsic, in particular the ability to sporulate, adaptation of pseudomonads, and the protective effects of biofilms. In these cases, "resistance" may be incorrectly used and "tolerance," defined as developmental or protective effects that permit microorganisms to survive in the presence of an active agent, may be more correct. Many of these reports of resistance have often paralleled issues including inadequate cleaning, incorrect product use, or ineffective infection control practices, which cannot be underestimated. Some acquired mechanisms (in particular with heavy-metal resistance) have also been shown to be clinically significant, but in most cases the results have been spec-

TABLE 16. Viral classification and response to some disinfectants[a]

Viral group	Lipid envelope[b]	Examples of viruses	Effects of disinfectants[c]	
			Lipophilic	Broad-spectrum
A	+	HSV, HIV, Newcastle disease virus, rabies virus, influenza virus	S	S
B	−	Non-lipid picornaviruses (poliovirus, Coxsackie virus, echovirus)	R	S
C	−	Other larger nonlipid viruses (adenovirus, reovirus)	R	S

[a] Data from reference 259; see also reference 444. For information on the inactivation of poliovirus, see reference 514.
[b] Present (+) or absent (−).
[c] Lipophilic disinfectants include QACs and chlorhexidine. S, sensitive; R, resistant.

ulative. Increased MICs have been confirmed, in particular for staphylococci. However, few reports have further investigated increased bactericidal concentrations or actual use dilutions of products, which in many cases contain significantly higher concentrations of biocides, or formulation attributes, which can increase product efficacy; in a number of cases, changes in the MICs have actually been shown not to be significant (9, 88, 89, 319, 428). Efflux mechanisms are known to be important in antibiotic resistance, but it is questionable if the observed increased MICs of biocides could have clinical implications for hard-surface or topical disinfection (423, 428). It has been speculated that low-level resistance may aid in the survival of microorganisms at residual levels of antiseptics and disinfectants; any possible clinical significance of this remains to be tested. With growing concerns about the development of biocide resistance and cross-resistance with antibiotics, it is clear that clinical isolates should be under continual surveillance and possible mechanisms should be investigated.

It is also clear that antiseptic and disinfectant products can vary significantly, despite containing similar levels of biocides, which underlines the need for close inspection of efficacy claims and adequate test methodology (183, 423, 428). In addition, a particular antiseptic or disinfectant product may be better selected (as part of infection control practices) based on particular circumstances or nosocomial outbreaks; for example, certain active agents are clearly more efficacious against gram-positive than gram-negative bacteria, and *C. difficile* (despite the intrinsic resistance of spores) may be effectively controlled physically by adequate cleaning with QAC-based products.

In conclusion, a great deal remains to be learned about the mode of action of antiseptics and disinfectants. Although significant progress has been made with bacterial investigations, a greater understanding of these mechanisms is clearly lacking for other infectious agents. Studies of the mechanisms of action of and microbial resistance to antiseptics and disinfectants are thus not merely of academic significance. They are associated with the more efficient use of these agents clinically and with the potential design of newer, more effective compounds and products.

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CHAPTER 2

Types of Antimicrobial Agents

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1 Introduction

Many different types of antimicrobial agents are now available and serve a variety of purposes in the medical, veterinary, dental and other fields (Russell *et al.*, 1984; Gorman & Scott, 1985; Gardner & Peel, 1986, 1991; Russell & Hugo, 1987; Russell, 1990a,b, 1991a,b; Russell & Gould, 1991a,b; Fleurette *et al.*, 1995; Marianos, 1995; Rossmore, 1995; Russell & Russell, 1995; Rutala, 1995a,b; Ascenzi, 1996a; Russell & Chopra, 1996). Subsequent chapters will discuss the factors influencing their activity and their role as disinfectants and antiseptics and as preservatives in a wide range of products or materials (Akers, 1984; Fels *et al.*, 1987; Eklund, 1989; Gould & Jones, 1989; Wilkins & Board, 1989; Russell & Gould, 1991a,b; Kabara & Eklund, 1991; Seiler & Russell, 1991). Lists of preservatives are provided by Denyer & Wallhäusser (1990) and by Hill (1995). Additional information is provided on their mechanism of action and on the ways in which microorganisms show resistance. Recent British Standards (1997a,b,c) describe tests for basic bactericidal and fungicidal activity and for hygienic handwashes.

The present chapter will concentrate on the antimicrobial properties and uses of the various types of antimicrobial agents. Cross-references to other chapters are made where appropriate. A comprehensive summary of inhibitory concentrations, toxicity and uses is provided by Wallhäusser (1984).

2 Phenols

The historical introduction (Chapter 1) and the papers by Hugo (1979, 1991) and Marouchoc (1979) showed that phenol and natural-product distillates containing phenols shared, with chlorine and iodine, an early place in the armoury of antiseptics. Today they enjoy a wide use as general disinfectants and as preservatives for a variety of manufactured products (Freney, 1995). The main general restriction is that they should not be used where they can contaminate foods.

As a result of their long history, a vast literature has accumulated dealing with phenol and its analogues, with bactericidal and bacteriostatic indices and phenol coefficient values measured against a large array of microorganisms. Unfortunately, many different parameters have been used to express their biocidal and biostatic power but the phenol coefficient (Chapter 4A) has probably been the most widely employed and serves as a reasonable cross-referencing cipher for the many hundreds of papers and reports written.

A feature of the work in the 1930s is the frequent exclusion of *Pseudomonas* species from the list of test organisms. At that time the pseudomonads were not regarded with the same degree of apprehension as they are today. The purpose of this section is not to repeat the mass of data but to take advantage of the passage of time and the present position to consider those phenolic derivatives which are likely to be found in common usage. The same accumulation of biological data has, however, enabled a reasonable assessment of the relationship between structure and activity in the phenol series to be compiled (Suter, 1941). The main conclusions from this survey were:

1 *para*-Substitutions of an alkyl chain up to six carbon atoms in length increases the antibacterial action of phenols, presumably by increasing the surface activity and ability to orientate at an interface. Activity falls off after this due to decreased water-solubility. Again, due to the

conferment of polar properties, straight chain *para*-substituents confer greater activity than branched-chain substituents containing the same number of carbon atoms.

2 Halogenation increases the antibacterial activity of phenol. The combination of alkyl and halogen substitution which confers the greatest antibacterial activity is that where the alkyl group is *ortho* to the phenolic group and the halogen *para* to the phenolic group. Russell *et al.* (1987) compared the activity of phenol, cresol and chlorocresol on wild-type and envelope mutant strains of *Escherichia coli* and found that chlorocresol was the most active against all strains and especially against deep rough mutants.

3 Nitration, while increasing the toxicity of phenol towards bacteria, also increases the systemic toxicity and confers specific biological properties on the molecule, enabling it to interfere with oxidative phosphorylation. This has now been shown to be due to the ability of nitrophenols to act as uncoupling agents. Studies (Hugo & Bowen, 1973) have shown that the nitro group is not a prerequisite for uncoupling, as ethylphenol is an uncoupler. Nitrophenols have now been largely superseded as plant protection chemicals, where at one time they enjoyed a large vogue, although 4-nitrophenol is still used as a preservative in the leather industry.

4 In the bis-phenol series, activity is found with a direct bond between the two C_6H_5–groups or if they are separated by $-CH_2-$, –S– or –O–. If a –CO–, –SO– or –CH(OH)– group separates the phenyl groups, activity is low. In addition, maximum activity is found with the hydroxyl group at the 2,2′- position of the bis-phenol. Halogenation of the bis-phenols confers additional biocidal activity.

2.1 Sources of phenols—the coal-tar industry

Most of the phenols that are used to manufacture disinfectants are obtained from the tar obtained as a by-product in the destructive distillation of coal. This process was carried out primarily, until the advent of North Sea gas, to produce coal gas, and is still used to produce coke and other smokeless fuels. Coal is heated in the absence of air and the volatile products, one of which is tar, condensed. The tar is fractionated to yield a group of products, which include phenols (called tar acids), organic bases and neutral products, such as alkyl naphthalenes, which are known in the industry as neutral oils. Phenols may be separated by extraction with alkali, from which they are regenerated by reaction with acid, e.g. carbon dioxide (CO_2) from flue gas.

The yield of tar acids from tar depends on the type of oven or retort used, the type of coal and the temperature of carbonization. Coal heated in a coke oven yields a tar with about 2% tar acid content; in the low-temperature process used to produce smokeless fuels, some 25% of the weight of tar consists of tar acids.

A typical but abridged analysis of the 50 or more phenolic substances produced is shown in Table 2.1. These figures are based on a low-temperature carbonization process.

The cresols consist of a mixture of 2-, 3- and 4-cresol. The 'xylenols' consist of the six isomeric dimethylphenols plus ethylphenols. The combined fraction, cresols and xylenols, is also available as a commercial product, which is known as cresylic acid. High-boiling tar acids consist of higher alkyl homologues of phenol, e.g. the diethylphenols, tetramethylphenols, methylethylphenols, together with methylindanols, naphthols and methylresorcinols, the latter being known as dihydrics. There may be traces of 2-phenylphenol. The chemical constituents of some of the phenolic components are shown in Fig. 2.1. Extended information on coal tars and their constituents is given in the *Coal Tar Data Book* (1965).

As tar distillation is a commercial process, it should be realized that there will be some overlap between fractions. Phenol is obtained at 99% purity. Cresol of the *British Pharmacopoeia* (1998) (2-, 3- and 4-cresols) must contain less than 2% of

Table 2.1 Typical analysis of a coal tar produced by the low-temperature carbonization process.

Phenol	Boiling range (°C)	Percentage
Phenol	182	7
Cresols	189–205	15
Xylenols	210–230	22
High-boiling tar acids	230–310	16

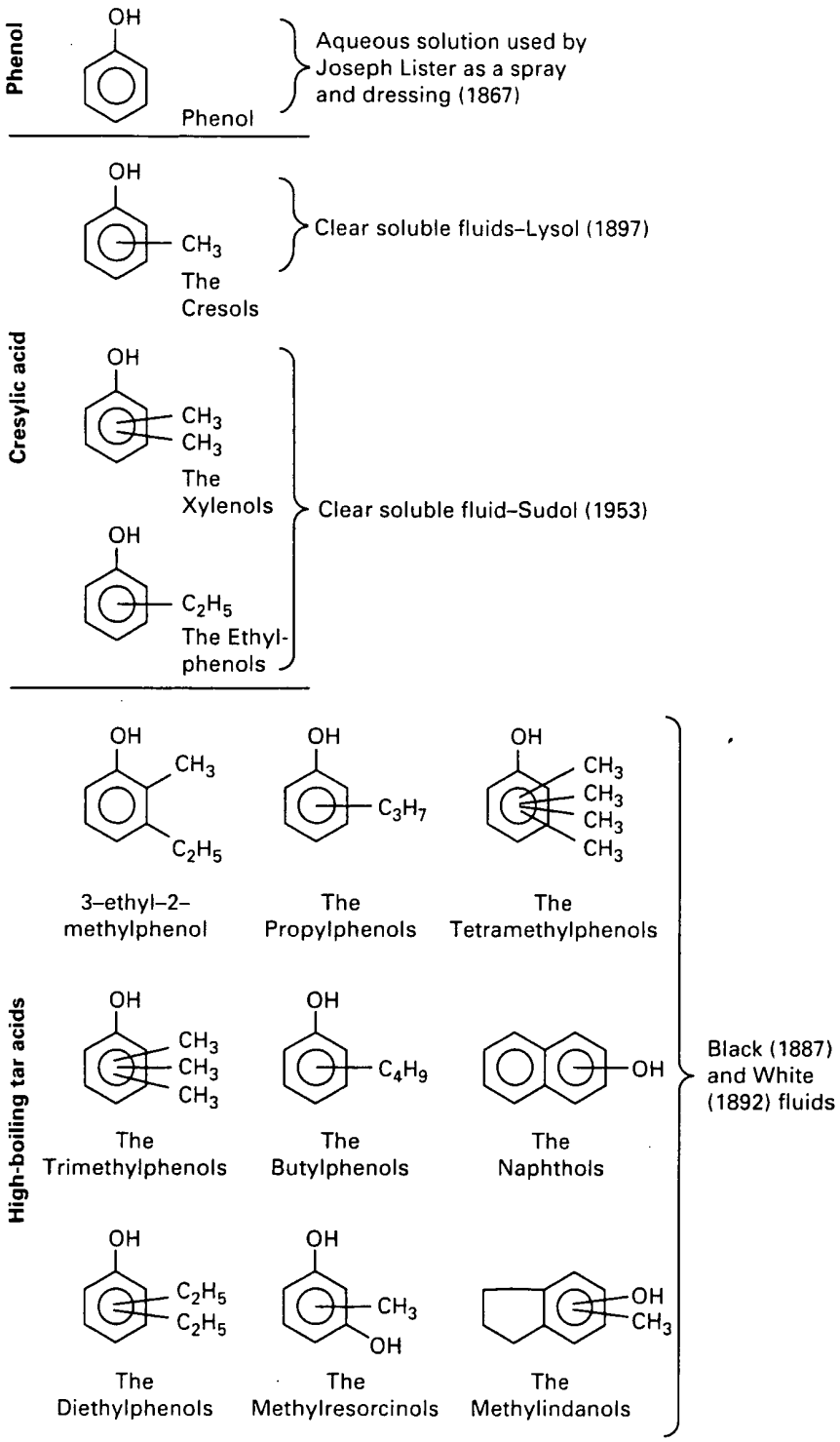


Fig. 2.1 Phenol, cresols, xylenols, ethylphenols and high-boiling tar acids.

phenol . A commercially mixed xylenol fraction contains no phenols or cresols but may contain 22 of the higher-boiling phenols. High-boiling tar acids may contain some of the higher-boiling xylenols, e.g. 3,4-xylenol (boiling-point (b.p.) 227°C).

Mention must be made of the neutral oil fraction, which has an adjuvant action in some of the formulated disinfectants to be considered below. It is devoid of biocidal activity and consists mainly of hydrocarbons, such as methyl- and dimethylnaphthalenes, *n*-dodecane, naphthalene,

tetramethylbenzene, dimethylindenes and tetrahydronaphthalene. Some tar distillers offer a neutral oil, boiling range 205–296°C, for blending with phenolics destined for disinfectant manufacture (see also Section 2.4.3).

2.2 Properties of phenolic fractions

The passage from phenol (b.p. 182°C) to the higher-boiling phenols (b.p. up to 310°C) is accompanied by a well-defined gradation in properties, as follows: water-solubility decreases, tissue trauma decreases, bactericidal activity increases, inactivation by organic matter increases. The ratio of activity against Gram-negative to activity against Gram-positive organisms, however, remains fairly constant, although in the case of pseudomonads, activity tends to decrease with decreasing water-solubility; see also Table 2.2.

2.3 Formulation of coal-tar disinfectants

It will be seen from the above data that the progressive increase in desirable biological properties of the coal-tar phenols with increasing boiling-point is accompanied by a decrease in water solubility. This presents formulation problems and part of the story of the evolution of the present-day products is found in the evolution of formulation devices.

The antiseptic and disinfectant properties of coal tar had been noted as early as 1815, and in 1844 a Frenchman called Bayard made an antiseptic powder of coal tar, plaster, ferrous sulphate and clay, an early carbolic powder. Other variations on this theme appeared during the first half of the nineteenth century.

In 1850, a French pharmacist, Ferdinand Le Beuf, living in Bayonne, prepared an emulsion of coal tar using the bark of a South American tree, the quillaia. This bark contained a triterpenoid glycoside with soap-like properties belonging to the class of natural products called saponins. By emulsifying coal tar, Le Beuf made a usable liquid disinfectant, which in the hands of the French surgeon, Lemaire, proved a very valuable aid to surgery. A 'solution' of coal tar prepared with quillaia bar was described in the *Pharmaceutical Codex* (1979); it would be interesting to know how many people attribute this formula to Le Beuf, who developed it 130 years ago. Quillaia is replaced by polysorbate 80 in formulae for coal-tar 'solutions' in the *British Pharmacopoeia* (1998).

In 1887 the use of soap and coal tar was first promulgated, and in 1889 a German experimenter, T. Damman, patented a product which was prepared from coal tar, creosote and soap and which involved the principle of solubilization. Thus, between 1850 and 1887, the basis for the formulation of coal-tar disinfectants had been laid and

Table 2.2 Phenol coefficients of coal-tar products against *Salmonella typhi* and *Staphylococcus aureus*.

Product and m.p., m. range (°C)	Phenol coefficient		Water-solubility (g/100 ml)
	S. typhi	*S. aureus*	
Phenol 182	1	1	6.6
Cresols 190–203	2.5	2.0	2.0
4-Ethylphenol 195	6	6	Slightly
Xylenols 210–230	5	4.5	Slightly
High-boiling tar acids 230–270	40	25	Insoluble
High-boiling tar acids 250–275	60	40	Insoluble

subsequent discoveries were either rediscoveries or modifications of these two basic themes of emulsification and solubilization. Better-quality tar acid fractions and products with clearer-cut properties aided the production of improved products.

In 1887 John Jeyes of Northampton patented a coal-tar product, the well-known Jeyes fluid, by solubilizing coal-tar acids with a soap made from the resin of pine trees and alkali. It is difficult, from the written history of the Jeyes Company (Palfreyman, 1977), to learn how John acquired the background knowledge for his product, but his brother, Philadelphus, was apprenticed to a pharmacist and might have supplied information on formulation.

In 1897, Engler and Pieckhoff in Germany prepared the first Lysol by solubilizing cresol with soap.

2.4 The modern range of solubilized and emulsified phenolic disinfectants

Black fluids are essential coal-tar fractions solubilized with soaps; white fluids are prepared by emulsifying tar fractions. Their composition as regards phenol content is shown in Fig. 2.1. The term 'clear soluble fluid' is also used to describe the solubilized products Lysol and Sudol.

2.4.1 Cresol and soap solution British Pharmacopoeia (BP) 1963 (Lysol)

This consists of cresol (a mixture of 2-, 3- and 4-cresols) solubilized with a soap prepared from linseed oil and potassium hydroxide; it forms a clear solution on dilution. Most vegetative pathogens, including mycobacteria, are killed in 15min by dilutions of Lysol ranging from 0.3 to 0.6%. Bacterial spores are much more resistant, and there are reports of the spores of *Bacillus subtilis* surviving in 2% Lysol for nearly 3 days. Even greater resistance has been encountered among clostridial spores. Lysol still retains the corrosive nature associated with the phenols and should be used with care. Both the method of manufacture and the nature of the soap used have been found to affect the biocidal properties of the product (Tilley & Schaffer, 1925; Berry & Stenlake, 1942). Rideal–Walker (RW) coefficients (British Standard (BS) 541: 1985; see Chapter 4A) are of the order of 2.

2.4.2 Xylenol-rich cresylic acid and soap solution (Sudol: Tenneco Organics Ltd, Avonmouth, Bristol)

By using a coal-tar fraction devoid of cresols but rich in xylenols and ethylphenols, a much more active but less corrosive product (Sudol) is obtained. Rideal–Walker coefficients as high as 7 gave been reported for this product. Sudol has a Chick–Martin coefficient (BS 808: 1986; see Chapter 4A) of 3.9 and is thus seen to be quite potent in the presence of organic matter; in fact, this phenol fraction seems to be the best of those normally used for disinfectant manufacture in retaining activity in the presence of organic debris. Other solubilized phenolic products in this category include Printol and Clearsol (also produced by Tenneco).

Sudol is active against *Mycobacterium tuberculosis* (phenol coefficient 6.3) and *Staphylococcus aureus* (phenol coefficient 6). The phenol coefficient against *Pseudomonas aeruginosa* is 4. It also possesses sporicidal activity: a 2% solution killed a suspension of *Clostridum perfringens* spores in 4 h; however, a suspension of *B. subtilis* spores needed 6 h in a 66% solution for inactivation. A full bacteriological protocol of activity against non-sporing organisms is given by Finch (1953).

Printol and Clearsol are similar to Sudol in general properties (all from Tenneco Organics, Avonmouth, BS11 0YT, UK). Another is Stericol (Sterling Health, Sheffield, S30 4YP, UK).

2.4.3 Black fluids

These are defined in a British Standard (BS 2462: 1986). They consist of a solubilized crude phenol fraction prepared from tar acids, of the boiling range 250–310°C (Fig. 2.1, Table 2.1).

The solubilizing agents used to prepare the black fluids of commerce include soaps prepared from the interaction of sodium hydroxide with resins (which contain resin acids) and with the sulphate and sulphonate mixture prepared by heating castor oil with sulphuric acid (called sulphonated castor oil or Turkey red oil).

Additional stability is conferred by the presence of coal-tar hydrocarbon neutral oils. These have already been referred to in Section 2.1 and comprise such products as the methylnaphthalenes, indenes and naphthalenes. The actual mechanism whereby they stabilize the black fluids has not been adequately explained; however, they do prevent crystallization of naphthalene present in the tar acid fraction. Klarmann & Shternov (1936) made a systematic study of the effect of the neutral oil fraction and also purified methyl- and dimethylnaphthalenes on the bactericidal efficiency of a coal-tar disinfectant. They prepared mixtures of cresol and soap solution (Lysol type) of the *United States Pharmacopoeia* with varying concentrations of neutral oil. They found, using a phenol coefficient-type test and *Salmonella typhi* as test organism, that a product containing 30% cresols and 20% neutral oil was twice as active as a similar product containing 50% cresols alone. However, the replacement of cresol by neutral oil caused a progressive decrease in phenol coefficient when a haemolytic streptococcus and *M. tuberculosis* were used as test organisms. The results were further checked using a pure 2-methylnaphthalene in place of neutral oil and similar findings were obtained. It is worth noting in parenthesis that, because of these divergent organism-dependent results, the authors argued against the use of *S. typhi* as the sole test organism in disinfectant testing.

Depending on the phenol fraction used and its proportion of cresylic acids to high-boiling tar acid, black fluids of varying RW coefficients reaching as high as 30 can be produced; however, as shown in Section 2.2, increasing biocidal activity is accompanied by an increasing sensitivity to inactivation by organic debris.

To obtain satisfactory products, the method of manufacture is critical and a considerable expertise is required to produce active and reproducible batches.

Black fluids give either clear solutions or emulsions on dilution with water, those containing greater proportions of higher phenol homologues giving emulsions. They are partially inactivated by the presence of electrolytes.

2.4.4 White fluids

These are also defined in BS 2462: 1986. They differ from the foregoing formulations in being emulsified, as distinct from solubilized, phenolic compounds. The emulsifying agents used include animal glue, casein and the carbohydrate extractable from the seaweed called Irish moss. Products with a range of RW coefficients may be manufactured by the use of varying tar-acid constituents.

As they are already in the form of an oil-in-water emulsion, they are less liable to have their activity reduced on further dilution, as might happen with black fluids if dilution is carried out carelessly. They are much more stable in the presence of electrolytes. As might be expected from a metastable system—the emulsion—they are less stable on storage than the black fluids, which are solubilized systems. As with the black fluids, products of varying RW coefficients may be obtained by varying the composition of the phenol. Neutral oils from coal tar may be included in the formulation.

An interesting account of the methods and pitfalls of manufacture of black and white fluids is given by Finch (1958).

2.5 Non-coal-tar phenols

As has been seen, the coal-tar (and to a lesser extent the petrochemical) industry yields a large array of phenolic products; phenol itself, however, is now made in large quantities by a synthetic process, as are some of its derivatives. Three such phenols, which are used in a variety of roles, are 4-tertiary octylphenol, 2-phenylphenol and 4-hexylresorcinol (Fig. 2.2).

2.5.1 4-Tertiary octylphenol

This phenol (often referred to as octylphenol) is a white crystalline substance, melting-point (m.p.) 83°C. The cardinal property in considering its application as a preservative is its insolubility in water, 1 in 60 000 (1.6×10^{-3}%). The sodium and potassium derivatives are more soluble. It is soluble in 1 in 1 of 95% ethanol and proportionally less soluble in ethanol containing varying proportions of water. It has been shown by animal-

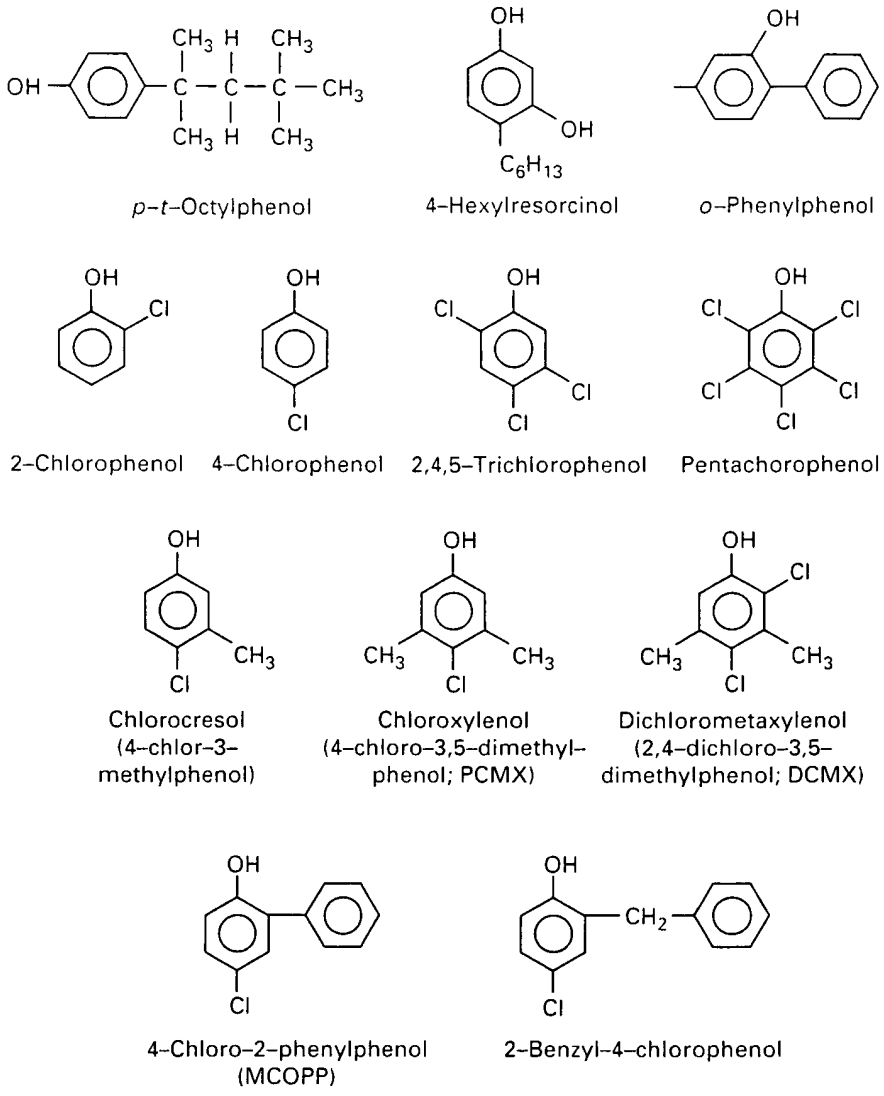


Fig. 2.2 Examples of phenolic compounds.

feeding experiments to be less toxic than phenol or cresol.

Alcoholic solutions of the phenol are 400–500 times as effective as phenol against Gram-positive organisms but against Gram-negative bacteria the factor is only one-fiftieth. Octylphenol is also fungistatic, and has been used as a preservative for proteinaceous products, such as glues and non-food gelatins. Its activity is reduced in the presence of some emulgents, a property that might render it unsuitable for the preservation of soaps and cutting oils.

2.5.2 2-Phenylphenol (2-phenylphenoxide)

This occurs as a white crystalline powder, melting at 57°C. It is much more soluble than octylphenol, 1 part dissolving in 1000 parts of water, while the sodium salt is readily soluble in water. It is both antibacterial and antifungal and is used as a preservative, especially against fungi, in a wide variety of applications. Typical minimal inhibitory concentrations (MICs, μg/ml) for the sodium salt are *E. coli*, 32; *S. aureus*, 32; *B. subtilis*, 16; *Pseudomonas fluorescens*, 16; *Aspergillus niger*, 4; *Epidermophyton* spp., 4; *Myrothecium verrucaria*, 2; *Trichophyton interdigitale*, 8. Many strains of *P. aeruginosa* are more resistant, requiring higher concentrations than those listed above for their inhibition.

Its main applications have been as ingredients in disinfectants of the pine type, as preservatives for cutting oils and as a general agricultural disinfectant. It has been particularly useful as a slimicide and fungicide in the paper and cardboard industry, and as an addition to paraffin wax in the

preparation of waxed paper and liners for bottle and jar caps.

2.5.3 *4-Hexylresorcinol*

This occurs as white crystalline needles (m.p. 67°C). It is soluble 0.5% in water but freely soluble in organic solvents, glycerol and glycerides (fixed oils). It is of low oral toxicity, having been used for the treatment of round- and whipworm infections in humans. It is used as a 0.1% solution in 30% glycerol as a skin disinfectant and in lozenges and medicated sweets for the treatment of throat infections.

2.6 Halo and nitrophenols

The general effect of halogenation (Fig. 2.2) upon the antimicrobial activity of phenols is to increase their activity but reduce their water solubility (Section 2.1). There is also a tendency for them to be inactivated by organic matter. The work on substituted phenols dates from the early twentieth century and was pioneered by Ehrlich and studied extensively by Klarmann *et al.* (1929, 1932, 1933).

To illustrate the effect of chlorination on the biocidal activity of phenols, RW coefficients are as follows: 2-chlorophenol, 3.6; 4-chlorophenol, 4; 3-chlorophenol, 7.4; 2,4-dichlorophenol, 13; 2,4,6-trichlorophenol, 22; 4-chloro-3-methylphenol, 13; 4-chloro-3,5-dimethylphenol, 30.

Chlorophenols are made by the direct chlorination of the corresponding phenol or phenol mixture, using either chlorine or sulphuryl chloride.

2.6.1 *2,4,6-Trichlorophenol*

This is a white or off-white powder, which melts at 69.5°C and boils at 246°C. It is a stronger acid than phenol with a pK_a (negative logarithm of acidic ionization constant; see Section 3.2) of 8.5 at 25°C. It is almost insoluble in water but soluble in alkali and organic solvents. This phenol has been used as a bactericidal, fungicidal and insecticidal agent. It has found application in textile and wood preservation, as a preservative for cutting oils and as an ingredient in some antiseptic formulations. Its phenol coefficient against *S. typhi* is 22 and against *S. aureus* 25.

2.6.2 *Pentachlorophenol (2-phenylphenoxide)*

A white to cream-coloured powder, m.p. 174°C, it can crystallize with a proportion of water, and is almost insoluble in water but soluble in organic solvents. Pentachlorophenol or its sodium derivative is used as a preservative for adhesives, textiles, wood, leather, paper and cardboard. It has been used for the in-can preservation of paints but it tends to discolour in sunlight. As with other phenols, the presence of iron in the products which it is meant to preserve can also cause discoloration.

2.6.3 *4-Chloro-3-methylphenol (chlorocresol)*

Chlorocresol is a colourless crystalline compound, which melts at 65°C and is volatile in steam. It is soluble 0.38% in water and readily soluble in ethanol, ether and terpenes. It is also soluble in alkaline solutions. Its pK_a at 25°C is 9.5. Chlorocresol is used as a preservative in pharmaceutical products and an adjunct in a former UK pharmacopoeial sterilization process called 'heating with a bactericide', in which a combination of heat (98–100°C) and a chemical biocide enabled a sterilization process to be conducted at a lower temperature than the more usual 121°C (see Chapter 3). Its RW coefficient in aqueous solution is 13 and nearly double this value when solubilized with castor oil soap. It has been used as a preservative for industrial products, such as glues, paints, sizes, cutting oils and drilling muds.

2.6.4 *4-Chloro-3,5-dimethylphenol (chloroxylenol; PCMX)*

Chloroxylenol is a white crystalline substance, melting at 155°C. It is soluble in water at 0.03% and readily soluble in ethanol, ether, terpenes and alkaline solutions. Its pK_a at 25°C is 9.7. It is used chiefly as a topical antiseptic, solubilized in a suitable soap solution and often in conjunction with terpineol or pine oil. Phenol coefficients for the pure compound were: *S. typhi*, 30; *S. aureus*, 26; *Streptococcus pyogenes*, 28; *Trichophyton rosaceum*, 25; *P. aeruginosa*, 11. It is not sporicidal and has little activity against the tubercle bacillus (in other words, it is a narrow-spectrum bactericide). It is also inactivated in the presence of or-

ganic matter. Its formulation into a solubilized, clear, liquid disinfectant will be considered below (Section 2.8). Its properties have been re-evaluated (Bruch, 1996).

2.6.5 2,4-Dichloro-3,5-dimethylphenol (dichloroxylenol; DCMX)

This is a white powder, melting at 94°C. It is volatile in steam and soluble in water at 0.02%. Although it is slightly less soluble than PCMX, it has similar properties and antimicrobial spectrum. It is used as an ingredient in pine-type disinfectants and in medicated soaps and hand scrubs.

2.6.6 Monochloro-2-phenylphenol

This is obtained by the chlorination of 2-phenylphenol and the commercial product contains 80% of 4-chloro-2-phenylphenol and 20% of 6-chloro-2-phenylphenol. The mixture is a pale straw-coloured liquid, which boils over the range 250–300°C. It is almost insoluble in water but may be used in the formulation of pine disinfectants, where solubilization is effected by means of a suitable soap.

2.6.7 2-Benzyl-4-chlorophenol

This occurs as a white to pink powder, which melts at 49°C. It has a slight phenolic odour and is almost insoluble in water. Suitably formulated by solubilization with vegetable-oil soaps, it has a wide biocidal spectrum, being active against Gram-positive and Gram-negative bacteria, viruses, protozoa and fungi.

2.6.8 Mixed chlorinated xylenols

A mixed chlorinated xylenol preparation can be obtained for the manufacture of household disinfectants by chlorinating a mixed xylenol fraction from coal tar.

2.6.9 Other halophenols

Brominated and fluorinated monophenols have been made and tested but they have not found extensive application.

2.6.10 Nitrophenols

Nitrophenols in general are more toxic than the halophenols. 3,5-Dinitro-o-cresol was used as an ovicide in horticulture, but the nitrophenol most widely used today is 4-nitrophenol, which is amongst a group of preservatives used in the leather manufacturing industry at concentrations of 0.1–0.5%. For a general review on the use and mode of action of the nitrophenols, see Simon (1953).

2.6.11 Formulated disinfectants containing chlorophenols

It will be seen from the solubility data recounted above that some formulation device, such as solubilization, already applied successfully to the more insoluble phenols, might be used to prepare liquid antiseptics and disinfectants based on the good activity and the low level of systemic toxicity and of the likelihood of tissue damage shown by chlorinated cresols and xylenols. Indeed, such a formula was patented in Germany in 1927, although the use of chlorinated phenols as adjuncts to the already existent coal-tar products had been mooted in England in the early 1920s.

In 1933, Rapps compared the RW coefficients of an aqueous solution and a castor-oil soap-solubilized system of chlorocresol and chloroxylenol and found the solubilized system to be superior by a factor of almost two. This particular disinfectant recipe received a major advance (also in 1933) when two gynaecologists, seeking a safe and effective product for midwifery and having felt that Lysol, one of the few disinfectants available to medicine at the time, was too caustic, made an extensive evaluation of the chloroxylenol–castor-oil product; their recipe also contained terpineol (Colebrook & Maxted, 1933). It was fortunate that this preparation was active against β-haemolytic streptococci, which are a hazard in childbirth, giving rise to puerperal fever. A chloroxylenol–terpineol–soap preparation is the subject of a monograph in the *British Pharmacopoeia (1998)*.

The bacteriology of this formulation has turned out to be controversial; the original appraisal indicated good activity against β-haemolytic

streptococci and *E. coli*, with retained activity in the presence of pus, but subsequent bacteriological examinations by experienced workers gave divergent results. Thus Colebrook in 1941 cast doubt upon the ability of solubilized chloroxylenol–terpineol to destroy staphylococci on the skin, a finding which was refuted by Beath (1943). Ayliffe *et al.* (1966) indicated that the product was more active against *P. aeruginosa* than *S. aureus*. As so often happens, however, *P. aeruginosa* was subsequently shown to be resistant and Lowbury (1951) found that this organism would actually multiply in dilutions of chloroxylenol–soap.

Although still an opportunistic organism, *P. aeruginosa* was becoming a dangerous pathogen, especially as more and more patients received radiotherapy or radiomimetic drugs, and attempts were made to potentiate the disinfectant and to widen its spectrum so as to embrace the pseudomonads. It had been well known that ethylenediamine tetraacetic acid (EDTA) affected the permeability of pseudomonads and some enterobacteria to drugs to which they were normally resistant (Russell, 1971a; Russell & Chopra, 1996) and both Dankert & Schut (1976) and Russell & Furr (1977) were able to demonstrate that chloroxylenol solutions with EDTA were most active against pseudomonads. Hatch & Cooper (1948) had shown a similar potentiating effect with sodium hexametaphosphate. This phenomenon may be worth bearing in mind when formulating hospital disinfectants.

2.6.12 Phenol

The parent compound C_6H_5OH (Fig. 2.1) is a white crystalline solid, m.p. 39–40°C, which becomes pink and finally black on long standing. It is soluble in water 1:13 and is a weak acid, pK_a 10. Its biological activity resides in the undissociated molecule. Phenol is effective against both Gram-positive and Gram-negative vegetative bacteria but is only slowly effective towards bacterial spores and acid-fast bacteria.

It is the reference standard for the RW and Chick–Martin tests for disinfectant evaluation (Chapter 4A). As has been mentioned (Chapter 1), it was used by Lister and others in pioneering work on antiseptic surgery. It finds limited application in medicine today, but is used as a preservative in such products as animal glues.

Although first obtained from coal tar, it is now largely obtained by synthetic processes, which include the hydrolysis of chlorobenzene of the high-temperature interaction of benzene sulphonic acid and alkali.

2.7 Pine disinfectants

As long ago as 1876, Kingzett took out a patent in Germany for a disinfectant deodorant made from oil of turpentine and camphor and which had been allowed to undergo oxidation in the atmosphere. This was marketed under the trade name Sanitas. Later, Stevenson (1915) described a fluid made from pine oil solubilized by a soap solution. This had a pine oil content of over 60°C.

The chief constituent of turpentine is the cyclic hydrocarbon pinene (Fig. 2.3). The odour of pinene, whether in turpentine or from pine oils made by distilling wood chips or leaves (needles) from various coniferous trees, has long held an association in the public's mind with freshness, cleanliness and a safe, disinfected environment, but the terpene hydrocarbons, of which pinene is but one example, have little or no biocidal activity.

The terpene alcohol terpineol (Fig. 2.3), which may be produced synthetically from pinene or turpentine via terpin hydrate, or in 80% purity by steam-distilling pine-wood fragments, is another ingredient of pine disinfectants and has already been exploited as an ingredient of the Colebrook

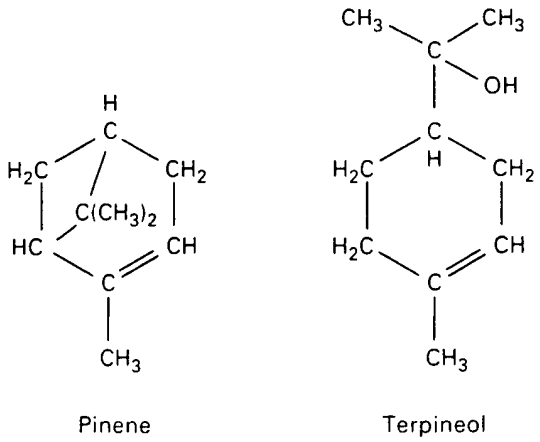


Fig. 2.3. Pinene and terpineol.

& Maxted (1933) chloroxylenol formulation. Unlike pinene, it possesses antimicrobial activity in its own right and it shares with pinene the property of modifying the action of phenols in solubilized disinfectant formulations, although not in the same way for all microbial species. An interesting experiment by Moore & Walker (1939) showed how the inclusion of varying amounts of pine oil in a PCMX/soap formulation modified the phenol coefficient of the preparation, depending on the test organism used.

Pine oil concentrations of from 0% to 10% caused a steady increase in the phenol coefficient from 2.0 to 3.6 when the test organism was *S. typhi*. With *S. aureus* the value was 0% pine oil, 0.6; 2.5% pine oil, 0.75; thereafter the value fell, having a value of only 0.03 with 10% oil, a pine-oil concentration which gave the maximum *S. typhi* coefficient. In this respect, pinene and terpineol may be compared with the neutral oils used in the coal-tar phenol products (Section 2.4.3), but it should be remembered that terpineol possesses intrinsic biocidal activity.

Terpineol is a colourless oil, which tends to darken on storing. It has a pleasant hyacinth odour and is used in perfumery, especially for soap products, as well as in disinfectant manufacture. A series of solubilized products has been marketed, with 'active' ingredients ranging from pine oil, pinene through terpineol to a mixture of pine oil and/or terpineol and a suitable phenol or chlorinated phenol. This gave rise to a range of products, extending from those which are really no more than deodorants to effective disinfectants.

Unfortunately there has been a tendency to ignore or be unaware of the above biocidal trends when labelling these varied products, and preparations containing a small amount of pine oil or pinene have been described as disinfectants. Attempts to remedy this situation have been made through the publication of a British Standard entitled *Aromatic Disinfectant Fluids* (BS 5197: 1976). The standard makes it clear that it specifies the requirements for a general-use disinfectant and does not imply use in hospitals or in other situations where there is a risk of infectious disease. The active ingredients, as stated in the standard, are substituted phenols together with pine oil or related terpenes and aromatic oils. A typical recipe for such a product would contain 4-chloro-3,5-xylenol, monochloro-2-phenylphenol, 2-phenylphenol, pine oil, terpineol and lime oil solubilized in water by means of potassium ricinoleate. Products with RW coefficients ranging from 3 to 10 may be produced, depending on the phenol content.

A further important requirement set out in the standard is that none of the products should be used at a use dilution more than 20 times the RW value, i.e. a product of RW 5 should never be used at dilutions greater than 1:100. Unrealistic use dilutions have contributed as much as uninformed formulation to the unreliability of some of these products.

It is very important that the labelling of this group of products should be carefully scrutinized before use and the mere possession of pine odour not used as the sole and final assessment for disinfectant potential.

2.8 Theory of solubilized systems

It will be apparent from the foregoing account that the art of obtaining aqueous solutions of relatively water-insoluble substances with the aid of soaps has been known since the late nineteenth century, when this technique was certainly applied to antiseptic systems.

Solubilization is achieved when anionic or cationic soaps aggregate in solution to form multiple particles of micelles, which may contain up to 300 molecules of the constituent species. These micelles are so arranged in an aqueous solution that the charged group is on the outside of the particle and the rest of the molecule is within the particle. It is in this part, often a hydrocarbon chain, that the phenols are dissolved, and hence solubilized, in an aqueous milieu.

The nature and antibacterial action of solubilized systems have intrigued many workers, notably Berry and his school, and Alexander and Tomlinson. The relationship between solubilization and antimicrobial activity was explored in detail by Bean & Berry (1950, 1951, 1953), who used a system consisting of 2-benzyl-4-chlorophenol (Section 2.6.7) and potassium laurate, and of 2,4-dichloro-3,5-dimethylphenol (Section 2.6.5) and potassium laurate. The advantage to a

fundamental understanding of the system is that potassium laurate can be prepared in a pure state and its physical properties have been well documented. 2-Benzyl-4-chlorophenol is almost insoluble in water and the antimicrobial activity of a solubilized system containing it will be uncomplicated by a residual water-solubility. The concepts were then extended to chlorocresol.

A plot of weight of solubilized substance per unit weight of solubilizer against the concentration of solubilizer at a given ratio of solubilized substance to solubilizer usually shows the type of curve illustrated in Fig. 2.4, curve OXYZ. Above the line OXYZ a two-phase system is found; below the curve a one-phase system consequent upon solubilization is obtained. Upon this curve has been superimposed a curve (O'ABC) which illustrates the change in bactericidal activity of such a system which is found if the solubilized substance possesses antibacterial activity. Such data give some indication of the complex properties of solubilized systems, such as Lysol and Roxenol. Bactericidal activity at O' is no more than that of the aqueous solution of the bactericide. The increase (O'–A) is due to potentiation of the action

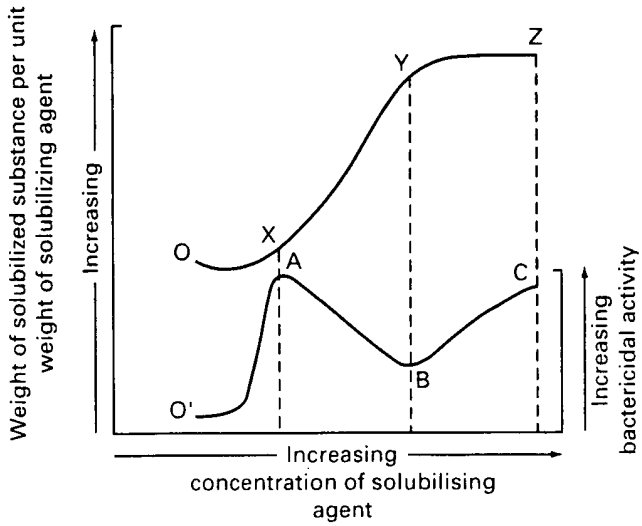


Fig. 2.4 The relationship between solubilization and antibacterial activity in a system containing a constant ratio of solubilized substance to solubilizer and where the solubilized substance possesses low water-solubility. *Curve OXYZ*, weight of solubilized substance per unit weight of solubilizing agent plotted against the concentration of solubilizing agent. *Curve O'ABC*, bactericidal activity of the system.

of the bactericide by unassociated soap molecules. At A, micelle formation and solubilization begin and thereafter (A–B) activity declines because, it has been suggested, the size of the micelle increases; the amount of drug per micelle decreases, and this is accompanied by a corresponding decrease in the toxicity of the system. However, at B an increase in activity is again found, reaching a maximum at C. This has been explained by the fact that at B, although increase in micellar size no longer occurs, increase in micellar number does, hence the gradual increase in activity.

The lethal event at cell level has been ascribed to an adsorption of the micelles by the bacterial cell and a passage of the bactericide from the micelle on to and into the bacterial cell. In short, this theory postulates that the bactericidal activity is a function of the concentration of the drug in the micelle and not its total concentration in solution. This was held to be the case for both the highly insoluble benzylchlorophenol and the more water-soluble chlorocresol (Bean & Berry, 1951, 1953). Alexander & Tomlinson (1949), albeit working with a different system, suggest a possible alternative interpretation. They agree that the increase, culminating at A, is due to the potentiation of the action of phenol by the solubilizing agent, which because it possesses detergent properties acts by disrupting the bacterial membrane, thereby permitting more easy access of the drug into the cell. The decline (A–B), however, was thought to be due to the removal of drug from the aqueous milieu into the micelles, thereby decreasing the amount available for reacting with the cell. They reject the notion that a drug-bearing micelle is lethal and capable itself of adsorption on the cell and passing its drug load to the cell, and declare that the activity of this system is a function of the concentration of bactericide in the aqueous phase. It must also be pointed out that high concentrations of soaps may themselves be bactericidal (reviewed by Kabara, 1978) and that this property could explain the increase in activity noted between B and C.

The above is only an outline of one experimental system in a very complex family. For a very complete appraisal together with further patterns of interpretation of experimental data of the problem, the papers of Berry *et al.* (1956) and

Berry & Briggs (1956) should be consulted. Opinion, however, seems to be settling in favour of the view that activity is a function of the concentration of the bactericide in the aqueous phase. Indeed, Mitchell (1964), studying the bactericidal activity of chloroxylenol in aqueous solutions of cetomacrogol, has shown that the bactericidal activity here is related to the amount of chloroxylenol in the aqueous phase of the system. Thus a solution which contained, as a result of adding cetomacrogol, 100 times as much of the bactericide as a saturated aqueous solution was no more bactericidal than the saturated aqueous solution. Here again, this picture is complicated by the fact that non-ionic surface-active agents, of which cetomacrogol is an example, are known to inactivate phenols (Beckett & Robinson, 1958).

2.9 The bis-phenols

Hydroxy halogenated derivatives (Fig. 2.5) of diphenyl methane, diphenyl ether and diphenyl sulphide have provided a number of useful biocides active against bacteria, fungi and algae. They all seem to have low activity against *P. aeruginosa*, however, i.e. they show the '*Pseudomonas* gap'; they also have low water solubility and share the property of the monophenols in that they are inactivated by non-ionic surfactants.

Ehrlich and co-workers were the first to investigate the microbiological activity of the bis-phenols and published their work in 1906. Klarmann and Dunning and colleagues described the preparation and properties of a number of these compounds (Klarmann & von Wowern, 1929; Dunning *et al.*, 1931). A useful summary of this early work has been made by Suter (1941). Later, Gump & Walter (1960, 1963, 1964) and Walter & Gump (1962) made an exhaustive study of the biocidal properties of many of these compounds, especially with a view to their use in cosmetic formulations.

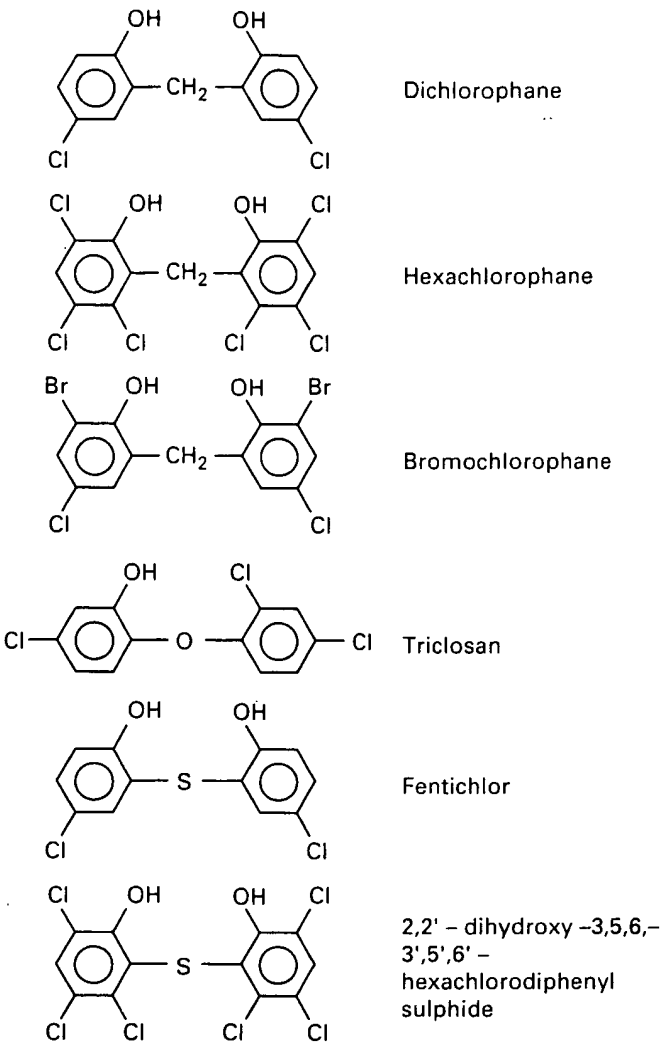


Fig. 2.5 Bis-phenols.

2.9.1 *Derivatives of dihydroxydiphenylmethane*

1 Dichlorophane, G-4,5,5′-dichloro-2,2′-dihydroxydiphenylmethane (Panacide, registered BDH, Poole, UK). This compound is active to varying degrees against bacteria, fungi and algae. It is soluble in water at 30 µg/ml but more soluble (45–80 g/100 ml) in organic solvents. The pK_a values at 25°C for the two hydroxyl groups are 7.6 and 11.6.

Typical killing concentrations in µg/ml in broth for bacteria at 25°C after 24 h incubation were: *S. aureus*, 2.5; *Streptococcus faecalis*, 7.5; *B. subtilis*, 7.5; *E. coli*, 10; *S. typhi*, 7.5; *P. aeruginosa* , 80; *Proteus mirabilis*, 50. The toxicity of the compound is low and it has been used for the treatment of tapeworm in humans and domestic animals at dose levels of 6 g on two successive days. It has also been used in the treatment of athlete's foot, indicating low skin-irritancy.

It has found application as a preservative for toiletries, textiles and cutting oils and to prevent

the growth of bacteria in water-cooling systems and humidifying plants. It is used as a slimicide in paper manufacture. It may be added to papers and other packing materials to prevent microbial growth and has been used to prevent algal growth in greenhouses.

2 Hexachlorophane, 2,2′-dihydroxy-3,5,6,3′,5′,6′-hexachlorodiphenylmethane, G11. This compound is almost insoluble in water but soluble in ethanol, ether and acetone and in alkaline solutions. The pK_a values are 5.4 and 10.9. Its mode of action has been studied in detail by Gerhardt, Corner and colleagues (Corner *et al.*, 1971; Joswick *et al.*, 1971; Silvernale *et al.*, 1971; Frederick *et al.*, 1974; Lee & Corner, 1975).

It is used mainly for its antibacterial activity but it is much more active against Gram-positive than Gram-negative organisms. Typical MICs (bacteriostatic) in μg/ml are: *S. aureus*, 0.9; *B. subtilis*, 0.2; *Proteus vulgaris*, 4; *E. coli*, 28; *P. aeruginosa*, 25.

It has found chief application as an active ingredient in surgical scrubs and medicated soaps and has also been used to a limited extent as a preservative for cosmetics. Its use is limited by its insolubility in water, its somewhat narrow antibacterial spectrum and by the fact that in the UK it is restricted by a control order made in 1973. In general, this order restricted the use of this product to 0.1% in human medicines and 0.75% in animal medicines. Its toxicity has restricted its use in cosmetic products, and the maximum concentration allowed is 0.1%, with the stipulation that it is not to be used in products for children or personal hygiene products.

3 Bromochlorophane, 3,3′-dibromo-5,5′-dichlor-2,2′-dihydroxydiphenylmethane. This product is soluble in water at 100 μg/ml and is markedly more active against Gram-positive organisms than bacteria. Strains of *S. aureus* are inhibited at from 8 to 11μg/ml, whereas 100 times these concentrations are required for *E. coli* and *P. aeruginosa*. It has been used as the active ingredient in deodorant preparations and toothpastes.

2.9.2 *Derivatives of hydroxydiphenylether*

1 Triclosan, 2,4,4′-trichlor-2′-hydroxydiphenylether (Irgasan, registered Ciga-Geigy Ltd, Basle, Switzerland). This derivative is only sparingly soluble in water but soluble in solutions of dilute alkalis and organic solvents. It inhibits staphylococci at concentrations ranging from 0.1 to 0.3 μg/ml. Paradoxically, a number of *E. coli* strains are inhibited over a similar concentration range. Most strains of *P. aeruginosa* require concentrations varying from 100 to 1000 μg/ml for inhibition. It inhibits the growth of several species of mould at from 1 to 30 μg/ml. It has a similar use potential to other bis-phenols and was the most widely used phenolic preservative reported in the survey by Richardson (1981), appearing in 52 formulations. It is used in some medicated soaps and hand-cleansing gels.

2.9.3 *Derivatives of diphenylsulphide*

1 Fenticlor, 2,2′-dihydroxy-5,5′-dichlorodiphenylsulphide. This chemical is a white powder, soluble in water at 30 μg/ml, but is much more soluble in organic solvents and oils. In common with its bisphenol cogeners, it shows more activity against Gram-positive organisms and a '*Pseudomonas* gap'. Typical inhibitory concentrations (μg/ml) are *S. aureus*, 2; *E. coli*, 100; *P. aeruginosa*, 1000. Typical inhibitory concentrations (μg/ml) for some fungi are: *Candida* spp., 12; *Epidermophyton interdigitale*, 0.4; *Trichophyton granulosum*, 0.4.

Fenticlor has found chief application in the treatment of dermatophytic conditions. It can cause photosensitization and this might limit its use as a cosmetic preservative. Its low water-solubility and narrow spectrum are further disadvantages, but it has potential as a fungicide. Its mode of action was described by Hugo & Bloomfield (1971a,b,c) and Bloomfield (1974).

2 Chlorinated analogue of fenticlor, 2,2′-dihydroxy-3,4,6,3′4′,6′-hexachlorodiphenylsulphide; 2,2′-thiobis(3,4,6-trichlorophenol). This is a more highly chlorinated analogue of fenticlor. It is almost insoluble in water. In a field test, it proved to be an effective inhibitor of microbial growth in cutting-oil emulsions.

An exhaustive study of the antifungal properties of hydroxydiphenylsulphides was made by Pfleger *et al.* (1949).

3 Organic and inorganic acids: esters and salts

3.1 Introduction

A large family of organic acids (Fig. 2.6), both aromatic and aliphatic, and one or two inorganic acids have found application as preservatives, more especially in the food industry. Some, for example benzoic acid, are also used in the preservation of pharmaceutical products; others (salicylic, undecylenic and again benzoic) have been used, suitably formulated, for the topical treatment of fungal infections of the skin.

Vinegar, containing acetic acid (ethanoic acid), has been known as long as alcohol, from which it would be formed by natural oxidation, and early on it had been found to act as a preservative. It was also used as a wound dressing. This application has been revived in the use of dilute solutions of acetic acid as a wound dressing where pseudomonal infections have occurred.

Hydrochloric and sulphuric acids are two mineral acids sometimes employed in veterinary disinfection. Hydrochloric acid at high concentrations is sporicidal and has been used for disinfecting hides and skin contaminated with anthrax spores. Sulphuric acid, even at high concentrations, is not sporicidal, but in some countries it is used, usually in combination with phenol, for the decontamination of floors, feed boxes and troughs (Russell & Hugo, 1987).

Citric acid is an approved disinfectant against foot-and-mouth virus. It also appears, by virtue of its chelating properties, to increase the permeability of the outer membrane of Gram-negative bacteria (Shibasaki & Kato, 1978; Ayres *et al.*, 1993) when employed at alkaline pH. Malic acid and gluconic acid, but not tartaric acid, can also act as permeabilizers at alkaline pH (Ayres *et al.*, 1993); see also Section 14.4.

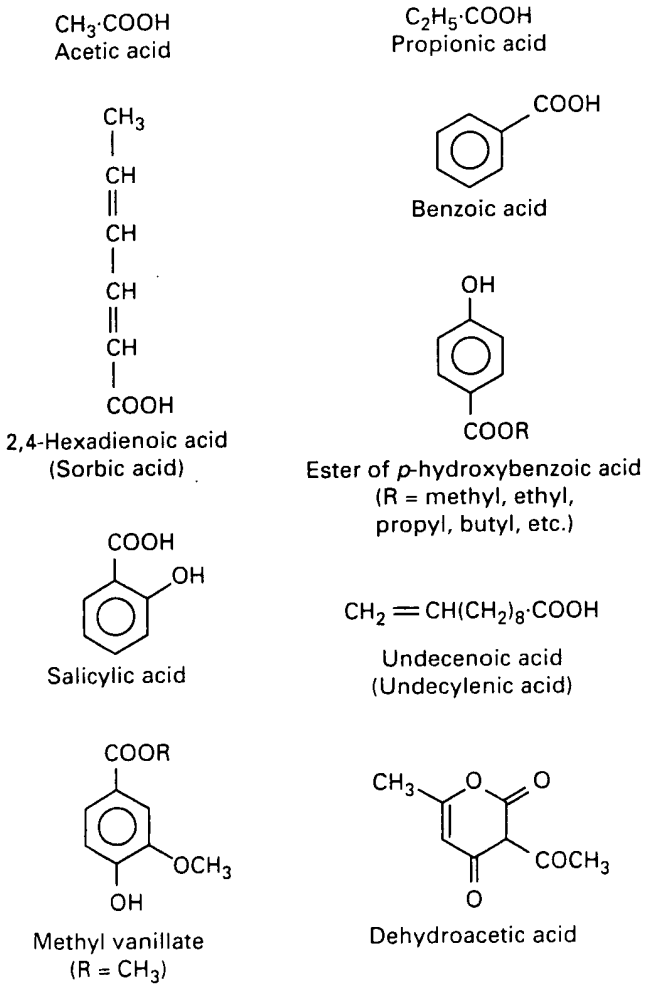


Fig. 2.6 Organic acids and esters.

3.2 Physical factors governing the antimicrobial activity of acids

At first sight, it might be thought that the special ability of acids to generate protons (hydrogen ions) when dissolved in water underlies their general toxicity; it is well known that acid conditions are inimical to the growth of many microorganisms. However, many successful antimicrobial acids are weak acids, i.e. they have dissociation constants between 10^{-3} and 10^{-5}; see below.

If an acid is represented by the symbol AH, then its ionization will be represented by A^-H^+. Complete ionization, as seen in aqueous solutions of mineral acids, such as hydrogen chloride (where AH = ClH), is not found in the weaker organic acids and their solutions will contain three components: A^-, H^+ and AH. The ratio of the concentration of these three components is called the ionization constant of that acid, K_a, and $K_a = A^- \times H^+/AH$. By analogy with the mathematical device used to define the pH scale, if the negative logarithm of K_a is taken, a number is obtained, running from about 0 to about 14, called pK_a. Some typical pK_a values are shown in Table 2.3.

An inspection of the equation defining K_a shows

Table 2.3 pK_a values of acids and esters used as antimicrobial agents.

Acid or esters	pK_a
Acetic (ethanoic) acid	4.7
Propionic (propanoic acid)	4.8
Sorbic acid (2,4-hexadienoic acid)	4.8
Lactic acid	3.8
Benzoic acid	4.2
Salicylic acid	3.0
Dehydroacetic acid	5.4
Sulphurous acid	1.8, 6.9
Methyl-*p*-hydroxybenzoic acid	8.5
Propyl-*p*-hydroxybenzoic acid	8.1

that the ratio A^-/AH must depend on the pH of the solution in which it is dissolved, and Henderson and Hasselbalch derived a relationship between this ratio and pH as follows:

$$\log \frac{A^-}{AH} = pH - pK_a$$

The application of this equation to the relative proportions of C_6H_5OOH and $C_6H_5COO^-$ in solutions of benzoic acid dissolved in buffers of varying pH is shown in Table 2.4. An inspection of the formula will also show that at the pH value equal to the pK_a value the product is 50% ionized. These data enable an evaluation of the effect of pH on the toxicity of organic acids to be made.

Typically it has been found that a marked toxic effect is seen only when the conditions of pH ensure the presence of the un-ionized molecular species AH. As the pH increases or, to put it in another way, the equilibrium $HA = HA^- + A^+$ moves to the right, the concentration of HA falls and the toxicity of the system falls; this may be indicated by a higher MIC, longer death time or higher mean single-survivor time, depending on the criterion of toxicity (i.e. antimicrobial activity) chosen.

Table 2.4 Effect of pH on ionization of benzoic acid, pK_a 4.19.

pH	Molecular form (C_6H_5COOH) (%)	Ionic form ($C_6H_5COO^-$) (%)
3.24	90	10
3.59	80	20
3.82	70	30
4.01	60	40
4.19	50	50
4.36	40	60
4.55	30	70
4.79	20	80
5.14	10	90

An inspection of Fig. 2.7 would suggest that HA is more toxic than A^-. However, an altering pH can alter the intrinsic toxicity of the environment. This is due to H^+ alone, the ionization of the cell surface, the activity of transport and metabolizing enzymes and the degree of ionization of the cell surface and hence sorption of the ionic species on the cell. Too simplistic a view, therefore, of the undoubted pH effect on the activity of weak acids must not be assumed. The ideal test organism would be one which is insensitive to changes in pH over a wide range.

Some few pages have been devoted to the above but in the authors' experience predictions for preservative ability of acids validated at one pH are rendered meaningless when such a preservative is added without further consideration to a formulation at a higher pH. The pK_a of the acid preservative should always be ascertained and any pH shift of 1.5 units or more on the alkaline side of this can be expected to cause progressive loss of activity quite sufficient to invalidate the originally determined performance. That pH modifies the antimicrobial effect of benzoic acid has been known for a long time (Cruess & Richert, 1929). For more detailed accounts of the effect of pH on the intensity of action of a large number of ionizable biocides, the papers of Simon & Blackman (1949) and Simon & Beeves (1952a,b) should be consulted.

3.3 Mode of action

The mode of action of acids used as food preservatives has been studied by Freese *et al.* (1973), Sheu *et al.* (1975), Krebs *et al.* (1983), Salmond *et al.* (1984), Eklund (1980, 1985, 1989), Sofos *et al.* (1986), Booth & Kroll (1989) Cherrington *et al.* (1990, 1991) and Russell (1992). Convincing evidence has been produced that many acid preservatives act by preventing the uptake of substrates which depend on a proton-motive force for their entry into the cell, in other words they act as uncoupling agents (Chapter 9).

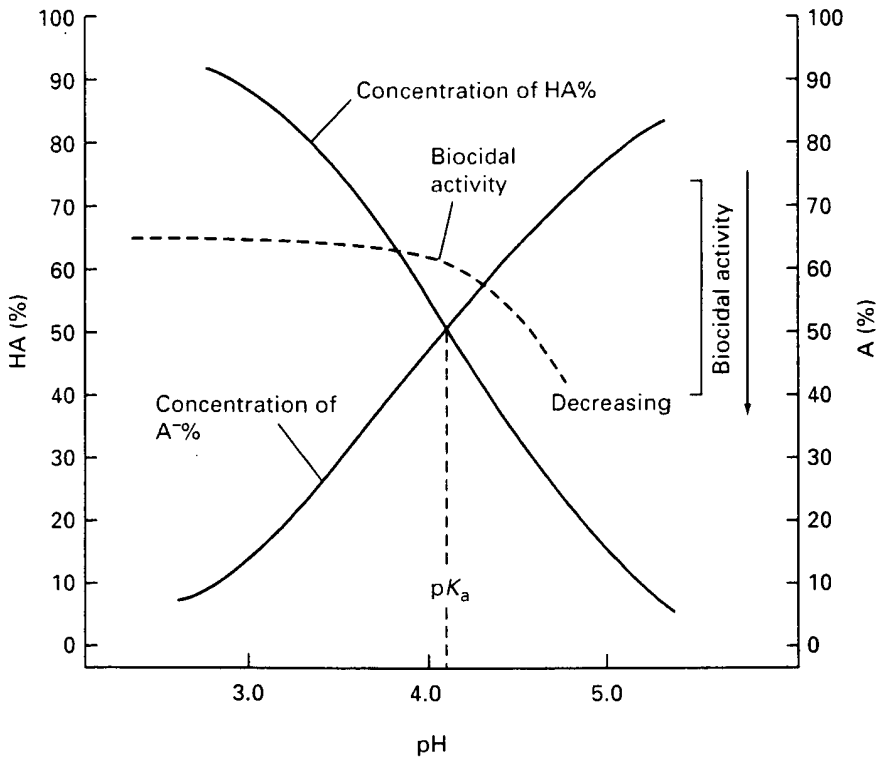


Fig. 2.7 A generalized diagram of the effect of pH on the ionization and biocidal activity of an acid (HA) of pK_a 4.1.

In addition to acids such as benzoic, acetic and propionic, the esters of *p*-hydroxybenzoic acid (the parabens) were also included in some of the above studies; they too acted as uncoupling agents but also inhibited electron transport.

Equally interesting were experiments on the pH dependence of the substrate uptake effect. The intensity of uptake inhibition by propionate, sorbate and benzoate declined between pH 5 and 7, while that induced by propyl-*p*-hydroxybenzoic acid (pK_a 8.5) remained constant over the same pH range. As has been stated, the growth-inhibitory effect of ionizable biocides shows pH dependence and this, as might be expected, is applicable to a biochemical effect upon which growth in turn depends. The total complement of compounds investigated by Freese *et al.* (1973) and Freese & Levin (1978) were acetic, benzoic, propionic, sorbic, caprylic and sulphurous acids and the methyl, propyl and heptyl esters of *p*(4)-hydroxybenzoic acid.

Organic acids, such as benzoic and sorbic, are deliberately used as preservatives. Acids such as acetic, citric and lactic are often employed as acidulants, i.e. to lower artificially the pH of foods. A low pK_a value is not the only significant feature of acidulants, however, since: (i) sorbate and acetate have similar pK_a values but the latter is a less potent preservative; (ii) organic acids used as preservatives are more potent inhibitors than other weak acids of similar pH; and (iii) weak organic acid preservatives are more effective inhibitors of pH homoeostasis than other acids of similar structure.

3.4 Individual compounds

3.4.1 Acetic acid (ethanoic acid)

This acid, as a diluted petrochemically produced compound or as the natural product vinegar, is used primarily as a preservative for vegetables, of which the onion (pickled onions) is the most familiar example. It is an ingredient of many sauces, pickles and salad cream, although the latter may need additional preservation. As it is a weak acid (pK_a 4.7), it is not likely to destroy the intracellular pectin of plant tissue, thus causing unsightly fragmentation.

Vinegars vary in strength but a wine vinegar may contain 8% of acetic acid. Vinegars made from acetic acid contain not less than 4% of acid, may be artificially coloured brown with caramel and must be clearly labelled as artificial. The

toxicity of vinegars and diluted acetic acid must rely to an extent on the inhibitory activity of the molecule itself, as solutions of comparable pH made from mineral acid do not exhibit the same preservative activity. A 5% solution of acetic acid contains 4.997% CH_3COOH and 0.003% H^+. As might be expected from the pK_a value, 4.7, the activity is rapidly lost at pH values above this value. This suggests that the acetate ion is less toxic than the undissociated molecule, although, as has been said, the concomitant reduction in hydrogen ion concentration must play some part in the reduction of toxicity. As has been stated, diluted 1–5% acetic acid has been used as a wound dressing where infection with *Pseudomonas* has occurred (Phillips *et al.*, 1968).

3.4.2 *Propionic acid*

This acid is employed almost exclusively as the sodium, and to a lesser extent the calcium, salt in the baking industry, where it is used to inhibit mould and bacterial growth in breads and cakes. It is particularly useful in inhibiting the growth of the spore-forming aerobe *Bacillus macerans*, which gives rise to an infestational phenomenon called ropy bread.

Manufacturers give explicit directions as to the amount to be used in different products, but in general 0.15–0.4% is added to the flour before processing. Other products that have been successfully preserved with propionates include cheeses and malt extract. In addition to foods, wrapping materials for foods have also been protected from microbial damage with the propionates.

3.4.3 *Undecanoic acid (undecylenic acid)*

This has been used either as such or as the calcium or zinc salt in the treatment of superficial dermatophytoses. It is usually applied in ointment form at concentrations of 2–15%.

3.4.4 *2,4-Hexadienoic acid (sorbic acid)*

This unsaturated carboxylic acid, which is also available as its potassium salt, is assimilated by mammals as food. It is effective against a wide range of microorganisms (Bell *et al.*, 1959) and has been used as the acid itself, or its potassium salt, at concentrations of 0.01–0.1% to preserve bakery products, soft drinks, alcoholic beverages, cheeses, dried fruits, fish, pickles, wrapping materials and pharmaceutical products. As with all acids, there is a critical pH, in this case 6.5, above which activity begins to decline. Again it is the undissociated acid which is the active antimicrobial species (Beneke & Fabian, 1955; Gooding *et al.*, 1955). Sorbic acid was believed to act by interfering with the functioning of the citric acid cycle (York & Vaughan, 1955; Palleroni & de Prinz, 1960).

Sorbic acid is known to interfere with the uptake of amino and oxo acids in *E. coli* and *B. subtilis*; it affects the proton-motive force in *E. coli* and accelerates the movement of H^+ ions from low media pH into the cytoplasm. It probably acts overall by dissipating ΔpH across the membrane and inhibiting solute transport. The membrane potential ($\Delta\psi$) is reduced but to a much smaller extent than ΔpH (Eklund, 1989; Cherrington *et al.*, 1991; Kabara & Eklund, 1991; Russell & Chopra, 1996). A combination of sorbic acid with monolaurin has been shown to be often more active than parabens or sorbic acid alone (Kabara, 1980).

3.4.5 *Lactic acid*

Lactic acid shares with some other hydroxyacids the interesting property of being able to destroy airborne microorganisms (Lovelock *et al.*, 1944; see also Section 19). A careful study of hydroxyacids, including lactic acid, as, air disinfectant was made by Lovelock (1948). Lactic acid was found to be a cheap, efficient aerial bactericide when sprayed into the area to be sterilized. It has, however, a slight irritant action on the nasal mucosa, which tends to limit its use. It could be used in emergencies for sterilizing glove boxes or hoods if other means of sterilization are not provided (see also Section 19).

Lactic acid in liquid form is less active than several other organic acids (Eklund, 1989) but nevertheless is used as an acidulant for low-pH foods and fruit juices (Russell & Gould, 1991a,b).

3.4.6 *Benzoic acid*

This organic acid occurs naturally in many natural

balsams and gums and these were used as preservatives early in human history. Benzoic acid, first shown to be antifungal in 1875, is a white crystalline powder, which is soluble 1:350 in water. It is used as a preservative for foods and pharmaceutical products, but is rapidly inactivated at pH values above 5.0 (Eklund, 1989; Kabara & Eklund, 1991; Russell & Gould, 1991b).

As with other preservatives, its activity may also be modified by the milieu in which it acts (Anderson & Chow, 1967; Beveridge & Hope, 1967. Resistance may develop (Ingram, 1959) and the acid may be metabolized by a contaminant it is meant to inhibit (Stanier *et al.*, 1950; Hugo & Beveridge, 1964; Stanier & Orston, 1973). In addition to its use as a preservative, benzoic acid has been combined with other agents for the topical treatment of fungal infections.

Benzoic acid, like many other compounds, inhibits swarming of *Bacillus* spp. (Thampuran & Surendran, 1996). Studies with benzoic acid derivatives have demonstrated that lipophilicity and pK_a are the two most important parameters influencing activity (Ramos-Nino *et al.*, 1996). The mode of action of benzoic acid is discussed in Chapter 9.

3.4.7 *Salicylic acid*

This is often used, in combination with benzoic acid and other antifungal agents, for the topical treatment of fungal infections. Salicylic acid has keratinolytic activity and in addition affects metabolic processes. For an account of the action of benzoic and salicylic acids on the metabolism of microorganisms, see Bosund (1962) and Freese *et al.* (1973).

3.4.8 *Dehydroacetic acid (DHA)*

Wolf (1950), looking at a general relationship between the structures of organic molecules possessing antimicrobial properties, noticed activity in a group of compounds containing an α,β unsaturated ketone residue. Wolf selected for study a series of 1,2 and 1,4 pyrones (Wolf & Westveer, 1950). Of these, 3-acetyl-6-methyl-1,2-*H*-pyran-2,4(3*H*)-dione (3-acetyl-4-hydroxy-6-methyl-2-acetyl-5-hydroxy-3-oxo-4-hexanoic acid-γ-lactone) or dehydroacetic acid showed especial promise.

Dehydroacetic acid is a white or light yellow, odourless, crystalline compound, which is soluble at less than 0.1% in water; the sodium salt is soluble to the extent of 33%. Typical inhibitory concentrations (%) of the latter for selected microorganisms are: *Aerobacter aerogenes*, 0.3; *Bacillus cereus*, 0.3; *Lactobacillus plantarum*, 0.1; *S. aureus*, 0.3; *P. aeruginosa*, 0.4; *A. niger*, 0.05; *Penicillium expansum*, 0.01; *Rhizopus nigricans*, 0.05; *T. interdigitale*, 0.005; *Saccharomyces cerevisiae*, 0.1. Extensive toxicological studies have indicated that the product is acceptable as a preservative for foods, cosmetics and medicines. The pK_a value of DHA is 5.4 but an inspection of pH/activity data suggests that activity loss above the pK_a value is not as great as with other preservative acids (propionic, benzoic) and indeed, in Wolf's 1950 paper, the MIC against *S. aureus* remained at 0.3% from pH 5 to 9. Loss of activity at alkaline pH values was, however, noted by Bandelin (1950) in his detailed study of the effect of pH on the activity of antifungal compounds, as would be predicted by the pK_a value.

Little was known about its mode of action, although Seevers *et al.* (1950) produced evidence that DHA inhibited succinoxidase activity in mammalian tissue, while Wolf & Westveer (1950) showed that it did not react with microbial –SH enzymes.

Dehydroacetic acid has found application in the preservation of foods, food wrappings, pharmaceuticals and toiletries. In the survey made by Richardson (1981) of the preservatives used in 18 500 cosmetic formulae, sodium dehydroacetate was used in 73 and dehydroacetic acid in 145.

3.4.9 *Sulphur dioxide, sulphites, bisulphites*

The fumes of burning sulphur, generating sulphur dioxide, have been used by the Greeks and Egyptians as fumigants for premises and food vessels to purify and deodorize. Lime sulphur, an aqueous suspension of elementary sulphur and calcium hydroxide, was introduced as a horticultural fungicide in 1803. Later, the salts, chiefly sodium, potassium and calcium, of sulphurous acid were used in wine and food preservation.

In addition to their antimicrobial properties, members of this group also act as antioxidants

helping to preserve the colour of food products, as enzyme inhibitors, as Maillard reaction inhibitors and as reducing agents (Gould & Russell, 1991).

A pH-dependent relationship exists in solution between the species SO_2, HSO_3^- and SO_3^{2-}. As the pH moves from acid to alkaline, the species predominance moves from SO_2, the toxic species, through HSO_3^- to SO_3^{2-}. Above pH 3.6, the concentration of SO_2 begins to fall, and with it the microbicidal power of the solution. It is postulated that SO_2 can penetrate cells much more readily than can the other two chemical species (Rose & Pilkington, 1989).

Yeasts and moulds can grow at low pH values, and hence the value of sulphites as inhibitors of fungal growth in acid environments, such as fruit juices. For reviews on the antimicrobial activity of sulphur dioxide, see Hammond & Carr (1976), Wedzicha (1984), Rose & Pilkington (1989) and Gould & Russell (1991).

3.4.10 Esters of p*-hydroxybenzoic acid (parabens)*

The marked pH-dependence of acids for their activity and the fact that the biocidal activity lay in the undissociated form led to the notion that esterification of an aromatic hydroxy carboxylic acid might give rise to compounds in which the phenolic group was less easily ionized.

Sabalitschka (1924) prepared a series of alkyl esters of *p*-hydroxybenzoic acid and tested their antimicrobial activity (Sabalitschka & Dietrich, 1926; Sabalitschka *et al.* 1926). This family of biocides, which may be regarded as either phenols or esters of aromatic hydroxy carboxylic acids, has stood the test of time and is today among the most widely used group of preservatives (Richardson, 1981).

As might be imagined for compounds which have been in use for over 60 years, there is an extensive literature. The esters usually used are the methyl, ethyl, propyl, butyl and benzyl compounds and are active over a wider pH range (4–8) than acid preservatives (Sokol, 1952), as has also been shown in biochemical experiments. Their pK_a values (8–8.5) compare with around 4 for preservative acids (Table 2.3). They have low water-solubility, which decreases in the order methyl–benzyl (Table 2.5). A paper which gives extensive biocidal data is that of Aalto *et al.* (1953). Table 2.5 shows typical data from the literature. Again it can be seen that activity increases from the methyl to the benzyl ester. The compounds show low systemic toxicity (Mathews *et al.*, 1956). Russell & Furr (1986a,b, 1987) and Russell *et al.* (1985, 1987) studied the effects of parabens against wild-type and envelope mutants of *E. coli* and *Salmonella typhimurium*, and found that, as the homologous series was ascended, solubility decreased but activity became more pronounced, especially against the deep rough strains.

In summary, it can be said that the parabens are generally more active against Gram-positive bacteria and fungi, including yeasts, than against Gram-negative bacteria, and in the latter *P. aeruginosa* is, as is so often seen, more resistant, especially to the higher homologues.

Hugo & Foster (1964) showed that a strain of *P. aeruginosa* isolated from a human eye lesion could metabolize the esters in dilute solution, 0.0343%, a solution strength originally proposed as a preservative vehicle for medicinal eye-drops. Beveridge & Hart (1970) verified that the esters could serve as a carbon source for a number of Gram-negative bacterial species. Rosen *et al.* (1977) studied the preservative action of a mixture of methyl (0.2%) and propyl (0.1%) *p*-hydroxybenzoic acid in a cosmetic lotion. Using a challenge test, they found that this concentration of esters failed to kill *P. aeruginosa*. It was part of their work indicating that these esters + imidazolindyl urea (Section 17.2.2) were ideal to provide a broad-spectrum preservative system, pseudomonads being successfully eliminated

It has been traditional to use these esters in mixtures, as for example in Rosen's experiments recounted above. The rationale for this might be seen in the preservation of water-in-oil emulsion systems, where the more water-soluble methyl ester protected the aqueous phase while the propyl or butyl esters might preserve the oil phase. This point is discussed by O'Neill *et al.* (1979).

Another factor which must be borne in mind when using parabens is that they share the property found with other preservatives containing a phenolic group of being inactivated by non-ionic

Table 2.5 Chemical and microbiological properties of esters of *p*-hydroxybenzoic acid.

Property*	Ester			
	Methyl	Ethyl	Propyl	Butyl
Molecular weight	152	166	180	194
Solubility in water (g/100 g) at 15°C)	0.16	0.08	0.023	0.005
K_w^o (arachis oil)	2.4	13.4	38.1	239.6
Log *P* (octanol:water)	1.96	2.47	3.04	3.57
MIC values (molar basis)†				
E. coli (wild type)	3.95×10^{-3}	2.7×10^{-3}	1.58×10^{-3}	1.03×10^{-3}
E. coli (deep rough)	2.63×10^{-3}	1.2×10^{-3}	2.78×10^{-4}	1.03×10^{-4}
MIC values (μg/ml)‡				
E. coli	800	560	350	160
P. aeruginosa	1000	700	350	150
Concentration (mmol/l) giving 50% inhibition of growth and uptake process in§				
E. coli	5.5	2.2	1.1	0.4
P. aeruginosa	3.6	2.8	>1.0	>1.0
B. subtilis	4.3	1.3	0.9	0.46

*K_w^o, partition coefficient, oil:water; *P*, partition coefficient, octanol:water.
†Russell *et al.* (1985).
‡El-Falaha *et al.* (1983).
§Eklund (1980).

surface agents. Hydrogen bonding between the phenolic hydrogen atom and oxygen residues in polyoxyethylated non-ionic surfactants is believed to be responsible for the phenomenon. Experiments to support this inactivation are described by Patel & Kostenbauder (1958), Pisano & Kostenbauder (1959) and Blaug & Ahsan (1961). Various ways of quenching paraben activity, including the use of polysorbates, are considered by Sutton (1996).

As has been stated, methyl and propyl parabens topped the league table of cosmetic preservatives (Richardson, 1981) and, provided their limitations are borne in mind, they form a very useful set of preservatives.

The mode of action of the parabens has been studied by Furr & Russell (1972a,b,c), Freese *et al.* (1973), Freese & Levin (1978), Eklund (1980, 1985, 1989) and Kabara & Eklund (1991). Haag & Loncrini (1984) have produced a comprehensive report of their antimicrobial properties.

3.4.11 Vanillic acid esters

The methyl, ethyl, propyl and butyl esters of vanillic acid (4-hydroxy-3-methoxy benzoic acid) possess antifungal properties when used at concentrations of 0.1–0.2%. These esters are not very soluble in water and are inactivated above pH 8.0. The ethyl ester has been shown to be less toxic than sodium benzoate and it has been used in the preservation of foods and food-packing materials against fungal infestation.

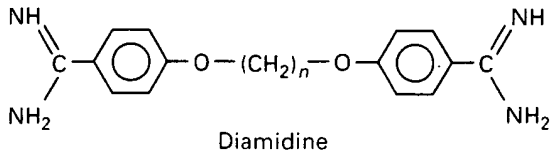
3.5 Regulations for the use of preservatives in foods

Certain of the foregoing substances described are used as food preservatives. The use of food preservatives is controlled by law in this and many other countries. Lloyd & Drake (1975) discuss problems associated with the addition of preservatives to foodstuffs. Legislative aspects have been comprehensively reviewed by Pollard (1991).

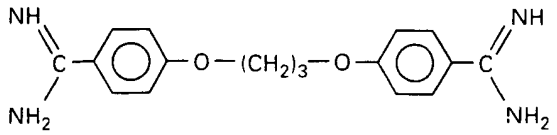
The special problems of food preservation are dealt with in Chapter 17 and of pharmaceutical products in Chapter 16, Lueck (1980) has published a detailed monograph on food preservatives, which includes a consideration of their history, uses, health aspects and regulatory status, while Tilbury (1980) discusses developments in food preservatives.

4 Aromatic diamidines

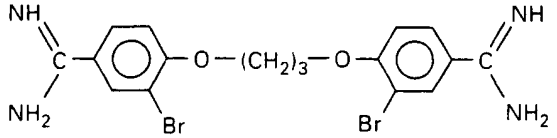
Diamidines are a group of organic compounds of which a typical structure is shown in Fig. 2.8. They were first introduced into medicine in the 1920s as possible insulin substitutes, as they lowered blood-sugar levels in humans. Because of these lowered levels, the notion was sustained that they might possess antitrypanosomal activity because of the exogenous requirement for glucose of this parasite. Later, they were found to possess an intrinsic trypanocidal activity not related to their action on blood sugar, and from this arose an investigation into their antimicrobial activity (Thrower & Valentine, 1943; Wien *et al.*, 1948). From these studies two compounds, propamidine and dibromopropamidine, emerged as useful antimicrobial compounds, being active against both bacteria and fungi.



Diamidine



Propamidine



Dibromopropamidine

Fig. 2.8 Typical structure of a diamidine; propamidine; dibromopropamidine.

4.1 Propamidine

Propamidine is 4,4′-diamidinophenoxypropane; in order to confer solubility on this molecule, it is usually supplied as the di(2-hydroxyethanesulphate), the isethionate. This product is a white hygroscopic powder, which is soluble in water, 1 in 5. Antimicrobial activity and clinical applications are described by Thrower & Valentine (1943). A summary of its antibacterial and antifungal activity is given in Table 2.6. Its activity is reduced by serum, by blood and by low pH values. Microorganisms exposed to propamidine quickly acquire a resistance to it by serial subculture in the presence of increasing doses. Methicillin-resistant *S. aureus* (MRSA) strains may show appreciable resistance to propamidine (see Chapter 10F). It is chiefly used in the form of a cream containing 0.15% as a topical application for wounds.

4.2 Dibromopropamidine

Dibromopropamidine is 2,2′-dibromo-4,4′-diamidinodiphenoxypropane, which is again usually supplied as the isethionate. It occurs as white crystals, which are readily soluble in water. Dibromopropamidine is active against Gram-positive, non-spore-forming organisms; it is less active against Gram-negative organisms and spore formers, but is active against fungi (Table 2.6). Resistance is acquired by serial subculture, and resistant organisms so induced also show a resistance to propamidine. Russell & Furr (1986b, 1987) found that Gram-negative bacteria present a permeability barrier to dibromopropamidine isethionate, and MRSA strains may be resistant to the diamidine (Chapter 10F). Its activity is reduced in acid environments and in the presence of blood and serum. It is usually administered as an oil-in-water cream emulsion containing 0.15% of the isethionate.

More detailed reviews on this group of compounds will be found in Hugo (1971) and Fleurette (1995).

5 Biguanides

Various biguanides show antimicrobial activity,

Table 2.6 Antimicrobial properties of propamidine and dibromopropamidine isethionates.

Microorganism	MIC (μg/ml) of	
	Propamidine isethionate*	Dibromopropamidine isethionate†
Staphylococcus aureus	1–16	1
Staphylococcus albus	6	
MRSA‡	800/100	
MRSE§	250–800	
Streptococcus pyogenes	0.24–4	1
Streptococcus viridans	1–4	2
Streptococcus faecalis	25	
Pseudomonas aeruginosa	250–400	32 (64)
Proteus vulgaris	125–400	128 (256)
Escherichia coli	64–100	4 (32)
Clostridium perfringens	3–32	512
Clostridium histolyticum	256	256
Shigella flexneri	32	8
Salmonella enteriditis	256	65
Salmonella typhimurium	256	64
Actinomyces kimberi	100	10
Actinomyces madurae	100	50
Actinomyces hominis	1000	1000
Trichophyton tonsurans	100	25
Epidermophyton floccosum	250	
Achorion schoenleinii	3.5	
Blastomyces dermatitidis	3.5	
Geotrichum dermatitidis	3.5	200
Hormodendron langevonii		500

*Data from various sources, including Wien *et al.* (1948).
†Data from Wien *et al.* (1948).
‡MRSA, methicillin-resistant *Staph. aureus* carrying *qacA/qacB* gene (data of Littlejohn *et al.*, 1992).
§MRSE, methicillin-resistant *Staph. epidermidis* (data of Leelaporn *et al.*, 1994).
Figures in parentheses denote bactericidal concentrations.

including chlorhexidine, alexidine and polymeric forms.

5.1 Chlorhexidine

Chlorhexidine (Fig. 2.9a) is one of a family of N^1, N^5-substituted biguanides which has emerged from extensive synthetic and screening studies, primarily by research workers at Imperial Chemical Industries (ICI) (Curd & Rose, 1946; Davies *et al.*, 1954; Rose & Swain, 1956). It is available as a dihydrochloride, diacetate and gluconate. At 20°C the solubilities of the dihydrochloride and diacetate are 0.06 and 1.9% w/v, respectively; the digluconate is freely soluble.

Chlorhexidine and its salts occur as white or faintly cream-coloured powders and are available in a number of pharmaceutical formulations. It is widely used combined with cetyltrimethylammonium bromide as a topical antiseptic (Savlon, Zeneca Ltd., Alderley Park, Macclesfield, Cheshire, UK).

Chlorhexidine has a wide spectrum of antibacterial activity against both Gram-positive and Gram-negative bacteria. Some bacteria, notably strains of *Proteus* and *Providencia* spp., may be highly resistant to the biguanide (Stickler *et al.*, 1983; Ismaeel *et al.*, 1986a,b; Russell, 1986; Baillie, 1987; see also Chapter 10A). It is not sporicidal (Shaker *et al.*, 1986, 1988a,b; Russell, 1990a,b, 1991b; Russell & Day, 1993; Ranganathan, 1996; Russell & Chopra, 1996). Chlorhexidine is not

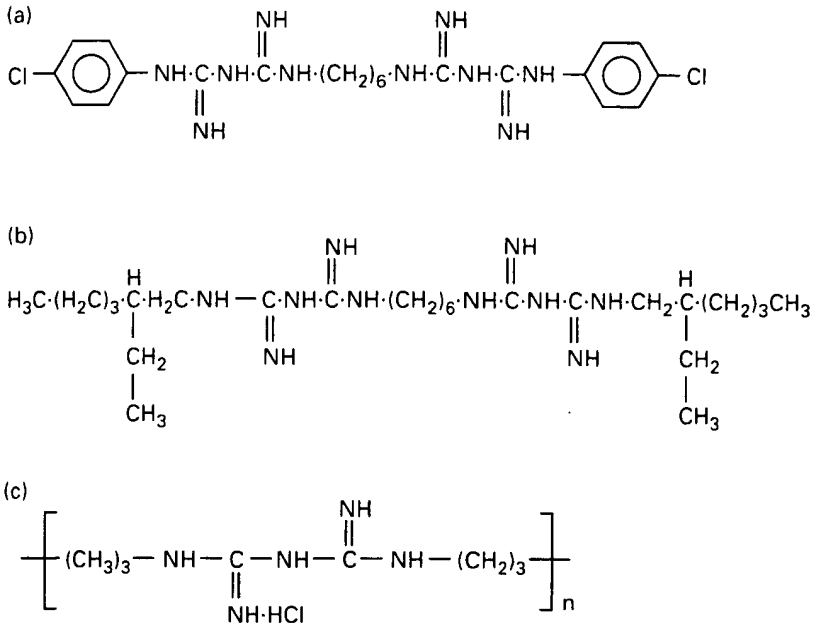


Fig. 2.9 Chlorhexidine (a), alexidine (b) and Vantocil 1B, a polymeric biguanide (c), in which mean n is 5.5.

lethal to acid-fast organisms, although it shows a high degree of bacteriostasis (Russell, 1995, 1996; Russell & Russell, 1995; Table 2.7). It is, however, tuberculocidal in ethanolic solutions and sporicidal at 98–100°C. A range of bacteriostatic and bactericidal values against a variety of bacterial species is shown in Tables 2.7 and 2.8, respectively.

Activity is reduced in the presence of serum, blood, pus and other organic matter. Because of its cationic nature, its activity is also reduced in the presence of soaps and other anionic compounds. Another cause of activity loss is due to the low solubility of the phosphate, borate, citrate, bicarbonate, carbonate or chloride salts. Any system which contains these anions will precipitate chlorhexidine.

Its main use is in medical and veterinary antisepsis (Holloway *et al.*, 1986). An alcoholic solution is a very effective skin disinfectant (Lowbury & Lilley, 1960). It is used in catheterization procedures, in bladder irrigation and in obstetrics and gynaecology. It is one of the recommended bactericides for inclusion in eye-drops and is widely used in contact-lens solutions (Gavin *et al.*, 1996). In the veterinary context (Russell & Hugo, 1987), chlorhexidine fulfils the major function of the application of a disinfectant of cows' teats after milking and can also be used as an antiseptic wound application. Chlorhexidine is

Table 2.7 Bacteriostatic activity of chlorhexidine against various bacterial species.

Organism	Concentration of chlorhexidine (μg/ml) necessary for inhibition of growth
Streptococcus lactis	0.5
Streptococcus pyogenes	0.5
Streptococcus pneumoniae	1.0
Streptococcus faecalis	1.0
Staphylococcus aureus	1.0
Corynebacterium diphtheriae	1.0
Salmonella typhi	1.67
Salmonella pullorum	3.3
Salmonella dublin	3.3
Salmonella typhimurium	5.0
Proteus vulgaris	5.0
Pseudomonas aeruginosa (1)	5.0
Pseudomonas aeruginosa (2)	5.0
Pseudomonas aeruginosa (3)	12.5
Enterobacter aerogenes	10
Escherichia coli	10+
Vibrio cholerae	3.3
Bacillus subtilis	0.5
Clostridium welchii	10
Mycobacterium tuberculosis	0.5
*Candida albicans**	5.0

Inoculum: one loopful of 24-h broth culture per 10 ml Difco heart–brain infusion medium.
Incubation: 24 h at 37°C.
*Yeast.
+Much higher than normally recorded.

Table 2.8 Bactericidal activity of chlorhexidine against various bacterial species.

Organism	Concentration of chlorhexidine (μg/ml)		
	To effect 99% kill	To effect 99.9% kill	To effect 99.99% kill
Staphylococcus aureus	8	14	25
Streptococcus pyogenes	–	–	50
Escherichia coli	6.25	10	20
Pseudomonas aeruginosa	25	33	60
Salmonella typhi	5	–	8

Inoculum: 10^5 in distilled water. Contact time: 10 min at room temperature. Neutralizer: egg-yolk medium.

also widely employed in the dental field (Gorman & Scott, 1985; Molinari, 1995; Cottone & Molinari, 1996).

Its mode of action has been studied by various authors (Hugo & Longworth, 1964a,b, 1965, 1966; Longworth, 1971, Hugo, 1978; Fitzgerald *et al.*, 1989, 1992a,b; Kuyyakanond & Quesnel, 1992; Barrett-Bee *et al.*, 1994; Russell & Day, 1996). ^{14}C-chlorhexidine gluconate is taken up very rapidly by bacterial (Fitzgerald *et al.*, 1989) and fungal (Hiom *et al.*, 1995a,b) cells. At lower concentrations, up to 200 μg/ml, it inhibits membrane enzymes and promotes leakage of cellular constituents; this is probably associated with bacteriostasis. As the concentration increases above this value, cytoplasmic constituents are coagulated and a bactericidal effect is seen (Chapter 9). Chlorhexidine has low oral toxicity and it may be administered for throat medication in the form of lozenges.

Extensive details on uses and application, together with relevant biocidal data, will be found in the booklet *Hibitane* (Imperial Chemical Industries, n.d.). Comprehensive surveys of its activity and uses have been published (Russell & Day, 1993; Reverdy, 1995a; Ranganathan, 1996).

5.2 Alexidine

Alexidine (Fig. 2.9b) is a bisbiguanide that possesses ethylhexyl end-groups as distinct from the chlorophenol end-groups found in chlorhexidine. Alexidine is considerably more active than chlorhexidine in inducing cell leakage from *E. coli*, and concentrations of alexidine (but not of chlorhexidine) above the MIC induce cell lysis (Chawner & Gilbert, 1989a,b). Alexidine has been recommended for use as an oral antiseptic and antiplaque compound (Gjermo *et al.*, 1973).

Unlike chlorhexidine, both alexidine and polyhexamethylene biguanide (PHMB) (Section 5.3) induce membrane lipid-phase separation and domain formation.

5.3 Polymeric biguanides

A novel compound, a polymer of hexamethylene biguanide (Fig. 2.9c), with a molecular weight of approximately 3000 (weight average), has found particular use as a cleansing agent in the food industry. Its properties have been described by Davies *et al.* (1968) under the trade name Vantocil 1B.

Polyhexamethylene biguanide is soluble in water and is usually supplied as a 20% aqueous solution. It is also soluble in glycols and alcohols but is insoluble in non-polar solvents, such as petroleum ethers or toluene. It inhibits the growth of most bacteria at between 5 and 25 μg/ml but 100 μg/ml is required to inhibit *P. aeruginosa* while *P. vulgaris* requires 250 μg/ml. It is less active against fungi; for example, *Cladosporium resinae*, which has been implicated as a spoilage organism in pharmaceutical products, requires 1250 μg/ml to prevent growth.

Polyhexamethylene biguanide is believed to gain access to Gram-negative bacteria by a mechanism of self-promotion through cation displacement from, predominantly, core lipopolysaccharide in the outer membrane (Gilbert *et al.*, 1990a). Antimicrobial activity of PHMB increases with increasing polymer length (Gilbert *et al.*, 1990b). It

is a membrane-active agent (Broxton *et al.*, 1983, 1984a,b; Woodcock, 1988), inducing phospholipid phase separation (Ikeda *et al.*, 1984). A complete loss of membrane function ensues, with precipitation of intracellular constituents leading to a bactericidal effect.

Because of the residual positive charges on the polymer, PHMB is precipitated from aqueous solutions by anionic compounds, which include soaps and detergents based on alkyl sulphates. It is also precipitated by detergent constituents, such as sodium hexametaphosphate, and in a strongly alkaline environment.

It finds use as a general sterilizing agent in the food industry, provided the surfaces to which it is applied are free from occlusive debris, a stricture that applies in all disinfection procedures. Because it is not a surface-active agent, it can be used in the brewing industry, as it does not affect head retention on ales and beers. Contact should be avoided with one commonly used material in food manufacture, anionic caramel, as this will, like other anionic compounds, inactivate the polymer. It has also been used very successfully for the disinfection of swimming pools. Apart from copper, which it tarnishes, this polymeric biguanide has no deleterious effect on most materials it might encounter in use.

Polyhexamethylene biguanide has activity against both the trophozite and the cyst forms of *Acanthamoeba castellanii* (Khunkitti *et al.*, 1996, 1997, 1998; see also Chapter 8A).

6 Surface-active agents

Surface-active agents (surfactants) have two regions in their molecular structure, one being a hydrocarbon water-repellent (hydrophobic) group and the other a water-attracting (hydrophilic or polar) group. Depending on the basis of the charge or absence of ionization of the hydrophilic group, surface-active agents are classified into anionic, cationic, non-ionic and ampholytic (amphoteric) compounds.

6.1 Cationic agents

Cationic surfactants possess strong bactericidal, but weak detergent, properties. The term 'cationic detergent' usually signifies a quaternary ammonium compound (QAC, onium compound), but this is not strictly accurate, as the smallest concentration at which a QAC is microbicidal is so low that its detergent activity is negligible (Davis, 1960).

Lawrence (1950), D'Aray & Taylor (1962a,b), Merianos (1991), Joly (1995) and Reverdy (1995b) have reviewed the surface-active quaternary ammonium germicides, and useful data about their properties and activity are provided by Wallhäusser (1984) and about their uses by Gardner & Peel (1986,1991) and Denyer & Wallhäusser (1990). Early references to their use are found in Jacobs (1916), Jacobs *et al.* (1916a,b) and Domagk (1935).

6.1.1 Chemical aspects

The QACs may be considered as being organically substituted ammonium compounds, in which the nitrogen atom has a valency of five, and four of the substituent radicals (R^1–R^4) are alkyl or heterocyclic radicals and the fifth (X^-) is a small anion (Fig. 2.10: general structure). The sum of the carbon atoms in the four R groups is more than 10. For a QAC to have a high antimicrobial activity, at least one of the R groups must have a chain length in the range C_8 to C_{18} (Domagk, 1935). Three of the four covalent links may be satisfied by nitrogen in a pyridine ring, as in the pyridinium compounds, such as cetylpyridinium chloride. This and the other important QACs are listed in Fig. 2.10. The cationic onium group may be a simple aliphatic ammonium, a pyridinium or piperidinium or other heterocyclic group (D'Arcy & Taylor, 1962b).

Apart from the monoquaternary compounds, monoquaternary derivatives of 4-aminoquinaldine (e.g. laurolinium) are potent antimicrobial agents, as are the bisquaternary compounds, such as hedaquinium chloride and dequalinium. These are considered in more detail in Section 10 (see also Fig. 2.22).

In addition to the compounds mentioned above, polymeric QACs are used as industrial biocides. One such compound is poly(oxyethylene(dimethylimino)ethylene)dichloride.

Organosilicon-substituted (silicon-bonded) qua-

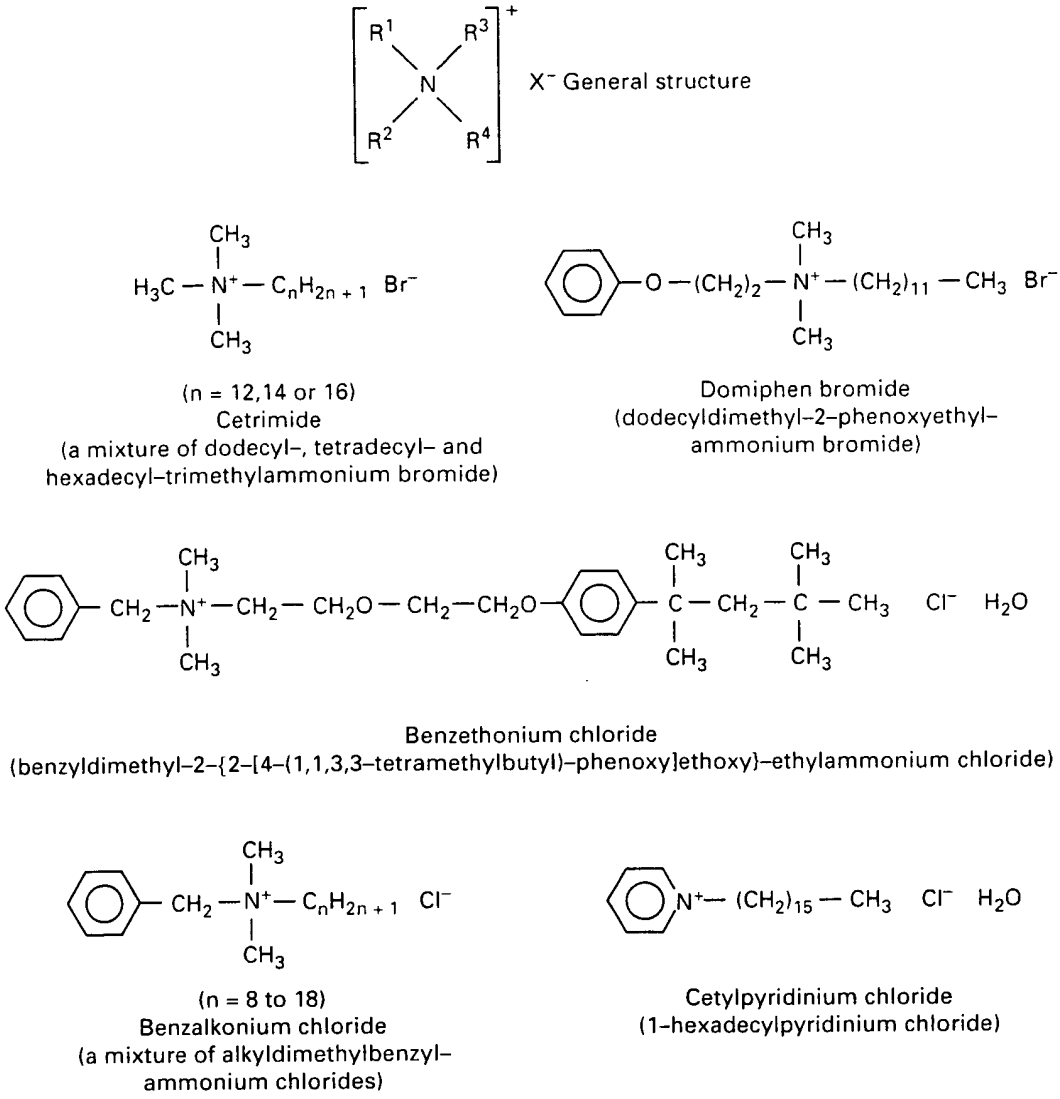


Fig. 2.10 General structure and examples of quaternary ammonium compounds (QACs).

ternary ammonium salts, organic amines or amine salts have been introduced recently. Compounds with antimicrobial activity in solution are also highly effective on surfaces. One such compound, 3-(trimethoxysily)propyloctadecyldimethyl ammonium chloride, demonstrates powerful antimicrobial activity while chemically bonded to a variety of surfaces (Malek & Speier, 1982; Speier & Malek, 1982). Schaeufele (1986) has pointed out that fatty alcohols and/or fatty acids, from both natural and synthetic sources, form the basis of the production of modern QACs, which have improved organic soil and increased hard-water tolerance.

6.1.2 *Antimicrobial activity*

As stated above, the antimicrobial properties of the QACs were first recognized in 1916, but they did not attain prominence until the work of Domagk in 1935. Early workers claimed that the QACs were markedly sporicidal, but the fallacy of this hypothesis has been demonstrated by improved testing methods. In particular, the experimental procedures devised by Davies (1949) are of considerable importance. He found that suspensions of *B. subtilis* spores were apparently sterilized after 1 h by various QACs when no precautions were made to prevent bacteriostasis in

the recovery medium; however, the inclusion in the recovery medium of Lubrol W (Bergan & Lystad, 1972; Mackinnon, 1974) showed the lack of sporicidal activity (Russell, 1971b). Weber & Black (1948) had earlier recommended the use of lecithin as a neutralizer for QACs. Lawrence (1948) showed that soaps and anionic detergents failed to inactivate QACs, and suggested suramin sodium for this purpose. British Standard 647 (1984) recommends lecithin (2%) solubilized with Lubrol W (3%), although Lubrol W itself may be toxic to streptococci, a point discussed more fully by Russell *et al.* (1979) and Russell (1981). Sutton (1996) describes appropriate neutralizing systems for QACs and other biocides; cyclodextrin (Simpson, 1992) may prove to be useful.

The QACs are primarily active against Gram-positive bacteria, with concentrations as low as 1 in 200 000 (0.0005%) being lethal; higher concentrations (*c.* 1 in 30 000 or 0.0033%) are lethal to Gram-negative bacteria (Hamilton, 1971), although *P. aeruginosa* tends to be highly resistant (Davis, 1962). Nevertheless, cells of this organism which are highly resistant to benzalkonium chloride (1 mg/ml, 0.1%) may still show ultrastructural changes when grown in its presence (Hoffman *et al.*, 1973). The QACs have a trypanocidal activity (reviewed by D'Arcy & Taylor, 1962b) but are not mycobactericidal (Sykes, 1965; Smith, 1968), presumably because of the lipid, waxy coat of these organisms. Gram-negative bacteria, such as *E. coli*, *P. aeruginosa* and *S. typhimurium*, exclude QACs, but deep rough mutants are sensitive (El-Falaha *et al.*, 1983; Russell & Furr, 1986a,b, Russell *et al.*, 1986; (Russell & Chopra, 1996). Contamination of solutions of QACs with Gram-negative bacteria has often been reported (Frank & Schaffner, 1976; Kaskow *et al.*, 1976).

Viruses are more resistant than bacteria or fungi to the QACs. This is clearly shown in the excellent review of Grossgebauer (1970), who points out that the QACs have a high protein defect, and that, whereas they are active against lipophilic viruses (such as herpes simplex, vaccinia, influenza and adenoviruses), they have only a poor effect against viruses (enteroviruses, e.g. polio, Coxsackie and Echo) that show hydrophilic properties. The QACs have, however, been demonstrated in laboratory tests to exhibit anti-human immunodeficiency virus (HIV) activity (Bond, 1995).

Activity of QACs against hepatitis B virus (HBV) has been shown by Prince *et al.* (1993). These findings led Bond (1995) to conclude that HIV and HBV are readily killed *in vitro* by a variety of biocidal agents, including QACs, but he emphasized the importance of the absence of organic matter.

The antiviral properties of QACs and other biocides are considered in detail in Chapter 6A.

The QACs possess antifungal properties, although they are fungistatic rather than fungicidal (for a review, see D'Arcy, 1971). This applies not only to the monoquaternary compounds, but also to the bisonium compounds, such as hedaquinium and dequalinium (Section 10; see also Chapter 9).

The Ferguson principle stipulates that compounds with the same thermodynamic activity will exert equal effects on bacteria. Weiner *et al.* (1965) studied the activity of three QACs (dodecyltrimethylammonium chloride, dodecyldimethylammonium chloride and dodecylpyridinium chloride) against *E. coli*, *S. aureus* and *Candida albicans*, and correlated these results with the surface properties of these agents. A clear relationship was found between the thermodynamic activity (expressed as a ratio of the surface concentration produced by a solution and the surface concentration at the critical micelle concentration (CMC)) and antibacterial activity.

Because most QACs are mixtures of homologues, Laycock & Mulley (1970) studied the antibacterial activity of mono- and multicomponent solutions, using the homologous series *n*-dodecyl, *n*-tetradecyl and *n*-hexadecyl trimethylammonium bromides individually, binary systems containing C_{12}/C_{14} or C_{14}/C_{16} mixtures, and a ternary mixture (centrimide) of the $C_{12}/C_{14}/C_{16}$ compounds. Antibacterial activity was measured as the concentrations needed to produce survivor levels of 1.0 and 0.01%; CMC was measured by the surface-tension method. In almost every instance, the thermodynamic activity (CMC/concentration to produce a particular survivor level) producing an equivalent biological response was reasonably constant, thereby supporting the Ferguson principle for these micelle-forming QACs.

The QACs are incompatible with a wide range

of chemical agents, including anionic surfactants (Richardson & Woodford, 1964), non-ionic surfactants, such as lubrols and tweens, and phospholipids, such as lecithin and other fat-containing substances. Benzalkonium chloride has been found to be incompatible with the ingredients of some commercial rubber mixes, but not with silicone rubber; this is important when benzalkonium chloride is employed as a preservative in multiple-dose eye-drop formulations (*Pharmaceutical Codex* 1994, *British Pharmacopoeia*, 1998).

Although non-ionic surfactants are stated above to inactivate QACs, presumably as a consequence of micellar formation (see Elworthy, 1976, for a useful description of micelles), nevertheless potentiation of the antibacterial activity of the QACs by means of low concentrations of non-ionic agents has been reported (Schmolka, 1973), possibly as a result of increased cellular permeability induced by the non-ionic surfactant (see Chapter 3 for a more detailed discussion).

The antimicrobial activity of the QACs is affected greatly by organic matter, including milk, serum and faeces, which may limit their usefulness in practice. The uses of the QACs are considered below (Section 6.1.3) and also in more general terms in Section 20. They are more effective at alkaline and neutral pH than under acid conditions. The action of benzalkonium chloride on *P. aeruginosa* is potentiated by aromatic alcohols, especially 3-phenylpropanol (Richards & McBride, 1973).

6.1.3 Uses

The QACs have many and varied uses. They have been recommended for use in food hygiene in hospitals (Kelsey & Maurer, 1972). Benzalkonium chloride has been employed for the preoperative disinfection of unbroken skin (0.1–0.2%), for application to mucous membranes (up to 0.1%) and for bladder and urethra irrigation (0.005%); creams are used in treating nappy (diaper) rash caused by ammonia-producing organisms, and lozenges for the treatment of superficial mouth and throat infections. In the UK, benzalkonium chloride (0.01% is one of four antimicrobial agents officially recognized as being suitable preservatives for inclusion in eye-drop preparations (*Pharmaceutical Codex* 1994, *British Pharmacopoeia*, 1993). Benzalkonium chloride is also widely used (at a concentration of 0.001–0.01%) in hard contact lens soaking (disinfecting) solutions; EDTA (see Section 13) at a concentration of 0.1% may be included to enhance its action (Kay, 1980). The QAC is too irritant to be used with hydrophilic soft (hydrogel) contact lenses because it can bind to the lens surface, be held within the water present in hydrogels and then be released into the eye (Davies, 1980).

Benzethonium chloride is applied to wounds as an aqueous solution (0.1%) and as a solution (0.2%) in alcohol and acetone for preoperative skin disinfection and for controlling algal growth in swimming pools.

Cetrimide is used for cleaning and disinfecting burns and wounds and for preoperative cleansing of the skin. For general disinfecting purposes, a mixture (Savlon) of cetrimide with chlorhexidine is often employed. At pH 6, but not at pH 7.2, this product may be liable to contamination with *P. aeruginosa* (Bassett, 1971). Solutions containing 1–3% of cetrimide are employed as hair shampoos (e.g. Cetavlon P.C., a concentrate to be diluted with water before use) for seborrhoea capitis and seborrhoeic dermatitis.

Cetylpyridinium chloride is employed pharmaceutically, for skin disinfection and for antiseptic treatment of small wound surfaces (0.1–0.5% solutions), as an oral and pharyngeal antiseptic (e.g. lozenges containing 1–2 mg of the QAC) and as a preservative in emulsions. Cosmetically (see also Quack, 1976), it is used at a concentration of between 0.1 and 0.5% in hair preparations and in deodorants; lower concentrations (0.05–0.1%) are incorporated into face and shaving lotions.

Several investigations have been made of the use of QACs in the disinfection of bedding and blankets (Schwabacher *et al.*, 1958; Gillespie & Robinson, 1959; Thomas *et al.*, 1959; Crewther & McQuade, 1964). Blankets and bedding comprise an important source of cross-infection in hospital wards. The bacteria associated with this cross-infection are usually non-sporing, and *S. aureus* is a particularly troublesome organism. Contamination of the air in a ward is very marked when beds are being made. The QACs were one of the first methods of disinfecting hospital woollen

blankets, which, however, are now rarely used.

In the veterinary context, the QACs have been used for the disinfection of automatic calf feeders and have been incorporated into sheep dips for controlling microbial growth in fleece and wool. They are not, however, widely used on farm sites because of the large amount of organic debris they are likely to encounter.

In general, then, the QACs are very useful disinfectants and pharmaceutical and cosmetic preservatives. Further information on their uses and antimicrobial properties is considered in Section 20 and in Chapters 3 and 16; see also BS 6471: 1984, BS 6424: 1950 and Reverdy (1995b).

6.2 Anionic agents

Anionic surface-active agents are compounds which, in aqueous solution, dissociate into a large complex anion, responsible for the surface activity, and a smaller cation. Examples of anionic surfactants are the alkali-metal and metallic soaps, amine soaps, lauryl ether sulphates (e.g. sodium lauryl sulphate) and sulphated fatty alcohols.

They usually have strong detergent but weak antimicrobial properties, except in high concentrations, when they induce lysis of Gram-negative bacteria (Salton, 1968). Fatty acids are active against Gram-positive but not Gram-negative bacteria (Galbraith *et al.*, 1971). More recent information will be found by consulting Kabara (1984).

6.3 Non-ionic agents

These consist of a hydrocarbon chain attached to a non-polar water-attracting group, which is usually a chain of ethylene oxide units, e.g. cetomacrogols. The properties of non-ionic surfactants depend mainly on the proportions of hydrophilic and hydrophobic groups in the molecule. Other examples include the sorbitan derivatives, such as the polysorbates (tweens).

The non-ionic surfactants are considered to have no antimicrobial properties. However, low concentrations of polysorbates are believed to affect the permeability of the outer envelopes of Gram-negative cells (Brown, 1975), which are thus rendered more sensitive to various antimicrobial agents. High concentrations of tweens overcome the activity of QACs, biguanides, parabens and phenolics. This is made use of in designing appropriate neutralizing agents (Russell *et al.*, 1979; Sutton, 1996) and is considered in more detail in Chapter 3.

6.4 Amphoteric (ampholytic) agents

Amphoteric agents are compounds of mixed anionic–cationic character. They combine the detergent properties of anionic compounds with the bactericidal properties of the cationic. Their bactericidal activity remains virtually constant over a wide pH range (Barrett, 1969) and they are less readily inactivated than QACs by proteins (Clegg, 1970). Examples of amphoteric agents are dodecyl-β-alanine, dodecyl-β-aminobutyric acid and dodecyl-di(aminoethyl)-glycine (Davis, 1960). The last-named belongs to the Tego series of compounds, the name Tego being a trade name (Goldschmidt, Essen).

The Tego compounds are bactericidal to Gram-positive and Gram-negative bacteria, and, unlike the QACs and anionic and non-ionic agents, this includes the mycobacteria (James, 1965; Croshaw, 1971), although the rate of kill of these organisms is less than that of the others (Block, 1983). Compounds based on dodecyl-di(aminoethyl)-glycine find use as disinfectants in the food industry (Kornfeld, 1966).

6.5 Betaines

Betaine itself is trimethylglycine. It is a natural constituent of beetroot and sugar beet and is obtained as a by-product of the sugar-beet industry.

Analogues, in which one of the methyl groups is replaced by a long-chain alkyl residue (Fig. 2.11), find application as detergents and as a basis for

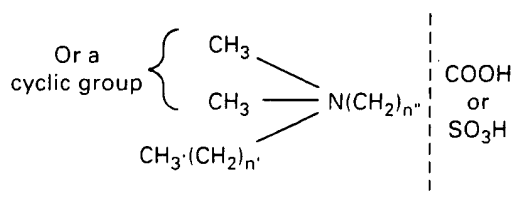


Fig. 2.11 General structure of betaines ($n' = 14-16$, $n'' = 1$ or 2).

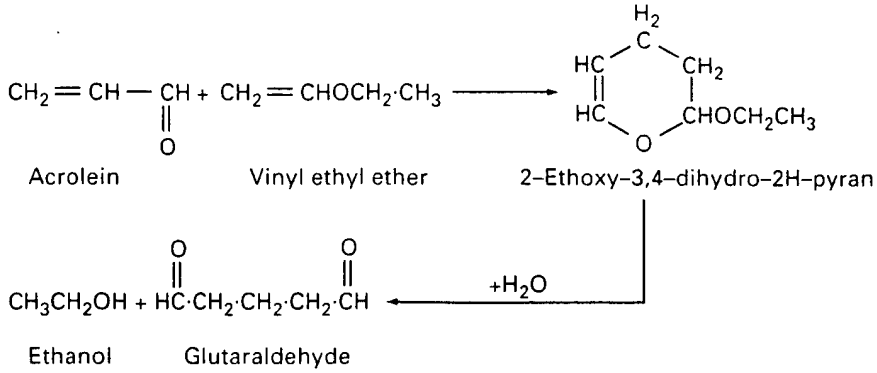


Fig. 2.12 Industrial production of glutaraldehyde.

solubilizing or emulsifying phenolic biocides. They have also been used in quaternary ammonium biocides (More & Hardwick, 1958) but are not considered as biocides *per se*.

Other chemical variants include the replacement of the –COOH group by $-SO_3H$ (Fig. 2.11) and of the two methyl groups by a ring system.

7 Aldehydes

Two aldehydes are currently of considerable importance as disinfectants, namely glutaraldehyde and formaldehyde, although others have been studied and shown to possess antimicrobial activity. Glyoxal (ethanedial), malonaldehyde (propanedial), succinaldehyde (butanedial) and adipaldehyde (hexanedial) all possess some sporicidal action, with aldehydes beyond adipaldehyde having virtually no sporicidal effects (Pepper & Chandler, 1963). Succinaldehyde is sometimes used as an antimicrobial agent, and this aspect is considered later (Section 7.3), in the form of Gigasept.

This section on aldehydes will deal mainly with glutaraldehyde and formaldehyde, although a 'new' aldehyde, o-phthalaldehyde, will also be considered briefly.

7.1 Glutaraldehyde (pentanedial)

7.1.1 Chemical aspects

Glutaraldehyde is a saturated 5-carbon dialdehyde with an empirical formula of $C_5H_8O_2$ and a molecular weight of 100.12. Its industrial production (Fig. 2.12) involves a two-step synthesis via an ethoxydihydropyran. Glutaraldehyde is usually obtained commercially as a 2, 25 or 50% solution of acidic pH, although for disinfecting purposes a 2% solution is normally supplied, which must be 'activated' (made alkaline) before use.

The two aldehyde groups may react singly or together to form bisulphite complexes, oximes, cyanohydrins, acetals and hydrazones. Polymerization of the glutaraldehyde molecule occurs by means of the following possible mechanisms.

1 The dialdehyde exists as a monomer, with an equilibrium between the open-chain molecule and the hydrated ring structure (Fig. 2.13a,b).

2 Ring formation occurs by an intramolecular mechanism, so that aqueous solutions of the aldehyde consist of free glutaraldehyde, the cyclic hemiacetal of its hydrate and oligomers of this is equlibrium (Fig. 2.13c).

3 Different types of polymers may be formed at different pH values, and it is considered that polymers in the alkaline range are unable to revert to the monomer, whereas those in the neutral and acid range revert easily (Boucher, 1974; Fig. 2.14).

Polymerization increases with a rise in pH, and above pH 9 there is an extensive loss of aldehyde groups. Glutaraldehyde is more stable at acid than alkaline pH; solutions at pH 8 and above generally lose activity within 4 weeks. Novel formulations have been produced, and continue to be designed, to overcome the problems of loss of stability (Babb *et al.*, 1980; Gorman *et al.*, 1980; Power, 1997).

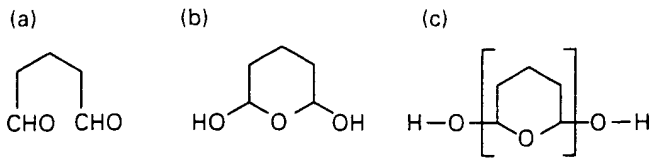


Fig. 2.13 (a) Free glutaraldehyde; (b) hydrated ring structure (cyclic hemiacetal of its hydrate); (c) oligomer.

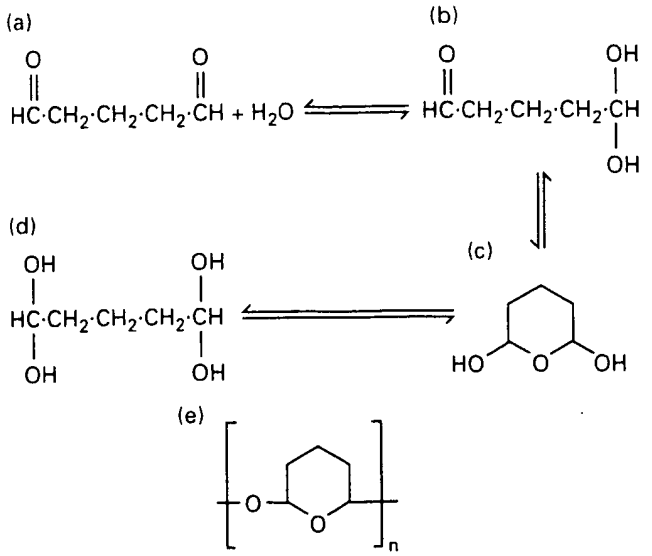


Fig. 2.14 (a) Open-chain molecule of glutaraldehyde; (b), (c) and (d) formation of several more stable 'polymers' (hydrated) in aqueous alkaline solution; (e) polymer with an acetal-like structure, in neutral and acid ranges (after Boucher, 1974).

7.1.2 *Interactions of glutaraldehyde*

Glutaraldehyde is a highly reactive molecule. It reacts with various enzymes (but does not sterically alter them to loss all activity) and with proteins; the rate of reaction is pH-dependent, increasing considerably over the pH range 4–9, and the reaction product is highly stable (Hopwood *et al.*, 1970). Glutarldehyde prevents dissociation of free ribosomes (Russell & Hopwood, 1976), but under the normal conditions of fixation (Hopwood, 1975) little reaction appears to occur between nucleic acids and glutaraldehyde. There is little published information on the possible reactions of glutaraldehyde and lipids (Russell & Hopwood, 1976).

7.1.3 *Microbicidal activity*

Glutaraldehyde possesses high microbicidal activity against bacteria and their spores, mycelial and spore forms of fungi and various types of viruses (Borick, 1968; Borick & Pepper, 1970). Although there was some doubt about its mycobactericidal potency, glutaraldehyde is now considered to be an effective antimycobacterial agent (Collins, 1986; Broadley *et al.*, 1991; Russell, 1994, 1996; Ascenzi, 1996c). Additional information is provided in Chapter 10D; see also Power (1997).

A summary of the antimicrobial efficacy of glutaraldehyde is presented in Table 2.9, which demonstrates the effect of pH on its activity. However, acid-based products are also available commercially which are claimed to be of equal activity to potentiated alkaline glutaraldehyde. Acid glutaraldehyde is itself an effective microbicide provided that long contact periods are used. The exact mechanism of action of the dialdehyde is unknown, but the fact that its rate of interaction with proteins and enzymes increases with increasing pH (Hopwood *et al.*, 1970; Russell & Munton, 1974) is undoubtedly of importance. The cross-linking mechanism is also influenced by time, concentration and temperature (Eager *et al.*, 1986; Bruch, 1991; Russell, 1994, 1996). Acid glutaraldehyde is a markedly inferior disinfectant to alkaline glutaraldehyde, but this discrepancy disappears with increasing temperature. Resistance development to glutaraldehyde is a late event in sporulation (Power *et al.*, 1988; Russell, 1990a,b, 1994, 1995; Knott *et al.*, 1995) and sodium hydroxide-induced revival of spores of *Bacillus* spp. has been demonstrated (Dancer *et al.*, 1989;

Table 2.9 Microbicidal activity of glutaraldehyde*.

Form of glutaraldehyde	Approximate pH value	Fungicidal activity†	Viricidal activity	Bactericidal activity‡	Sporicidal activity
Acid	4–5	Low	Low to high	Low	Low to very high
Alkaline	8	High	High	High	Reasonable to very high

*See also Gorman *et al.* (1980), Russell (1994) and Favero (1995).
†Use of low dialdehyde concentrations (0.01–0.02%); 2% solutions of acid and alkaline glutaraldehyde are both highly active against bacteria and probably viruses. A high-concentration (3.2%) glutaraldehyde solution is also available (Akamatsu *et al.*, 1997).
‡Activity of acid glutaraldehyde increases markedly with temperature and at *c.* 37°C its activity approaches that of alkaline glutaraldehyde. Acid glutaraldehyde may also be sporicidal at ambient temperatures, provided that long periods of time (*c.* 10 h) are used (Rutala *et al.*, 1993a,b).

Power *et al.*, 1989, 1990; Williams & Russell, 1992a,b).

Organic matter is considered to have no effect on the antimicrobial activity of the aldehyde. In view of the interaction of glutaraldehyde with the amino groups in proteins, this would appear to be a rather unusual finding. It is, however, true to state that it retains a considerable degree of activity in the presence of high levels of organic matter, such as 20% serum (A.D. Russell, unpublished data).

Dried spores are considerably more resistant to chemical disinfectants than are spores in suspension, and it would appear that glutaraldehyde is no exception. The use of the Association of Official Analytical Chemists (AOAC) test with dried spores of *B. subtilis* has shown that 2% alkaline glutaradehyde may require up to 10 h to achieve sterilization at 20°C (Rubbo *et al.*, 1967).

The antimicrobial activity of glutaraldehyde has been reviewed by Gorman *et al.* (1980), Bruch (1991), Russell (1994), Ascenzi (1996c) and Power (1997).

7.1.4 Uses of glutaraldehyde

The uses of glutaraldehyde as a fixative in electron microscopy, in leather tanning and biochemically have been discussed by Russell & Hopwood (1976). In a microbiological context, glutaraldehyde has been recommended for the disinfection/sterilization of certain types of medical equipment, notably cystoscopes and anaesthetic equipment.

Favero and Bond (1991) have rightly drawn attention to the differences between physical methods of sterilization and liquid chemical germicides and point out that 2% alkaline glutaraldehyde is capable of acting as a sterilizing agent but only after prolonged periods of contact. Bearing this comment in mind, glutaraldehyde has long been used for the high-level disinfection of endoscopes, although problems have arisen because of its toxicity. Glutaraldehyde has also been employed for the disinfection of arthroscopes and laparoscopes (Loffer, 1990).

As pointed out, alkaline glutaraldehyde is more active, but less stable, than the acid form. However, 2% activated alkaline glutaraldehyde should not be used continuously to disinfect endoscopes for 14 days after activation, although it is effective over this period if not repeatedly reused (Babb, 1993; Babb & Bradley, 1995). These authors recommend reuse for endoscopes provided that the concentration does not fall appreciably below 1.5%.

Problems in reusing glutaraldehyde are associated with accumulation of organic matter, dilution of disinfectant, change in product pH and difficulties in accurately assaying residual concentrations (Mbithi *et al.*, 1993; Rutala & Weber, 1995; Springthorpe *et al.*, 1995). Colour indicators are not always satisfactory (Power & Russell, 1988). Glutaraldehyde has been employed in the veterinary field for the disinfection of utensils and of premises (Russell & Hugo, 1987), but its potential mutagenic and carcinogenic effects (Quinn, 1987) make these uses hazardous to personnel. The main advantages claimed for glutaraldehyde are as follows: it has a broad spectrum of activity with a rapid microbicidal action, and it is non-corrosive to metals, rubber and lenses. Its toxicity (*vide supra*) remains a problem.

7.2 Formaldehyde (methanal)

Formaldehyde is used as a disinfectant as a liquid or vapour. Gaseous formaldehyde is referred to briefly in Section 18 and in more detail in Chapter 21. The liquid form will be considered mainly in this section.

The Health and Safety Executive of the UK has indicated that the inhalation of formaldehyde vapour may be presumed to pose a carcinogenic risk to humans. This indication must have considerable impact on the consideration of the role and use of formaldehyde and formaldehyde releasers in sterilization and disinfection processes.

7.2.1. Chemical aspects

Formaldehyde occurs as formaldehyde solution (formalin), an aqueous solution containing *c.* 34–38% w/w CH_2O. Methyl alcohol is present to delay polymerization. Formaldehyde displays many typical chemical reactions, combining with amines to give methylolamines, carboxylic acids to give esters of methylene glycol, phenols to give methylphenols and sulphides to produce thiomethylene glycols.

7.2.2 *Interactions of formaldehyde*

Formaldehyde interacts with protein molecules by attaching itself to the primary amide and amino groups, whereas phenolic moieties bind little of the aldehyde (Fraenkel-Conrat *et al.*, 1945). Subsequently, it was shown that formaldehyde gave an intermolecular cross-linkage of protein or amino groups with phenolic or indole residues.

In addition to interacting with many terminal groups in viral proteins, formaldehyde can also react extensively with the amino groups of nucleic acid bases, although it is much less reactive with deoxyribonucleic acid (DNA) than with ribonucleic acid (RNA) (Staehelin, 1958).

7.2.3 *Microbicidal activity*

Formaldehyde is a microbicidal agent, with lethal activity against bacteria and their spores, fungi and many viruses. Its first reported use as a disinfectant was in 1892. Its sporicidal action is, however, slower than that of glutaraldehyde (Rubbo *et al.*, 1967). Formaldehyde combines readily with proteins (Section 7.2.2) and is less effective in the presence of protein organic matter. Plasmid-mediated resistance to formaldehyde has been described, presumably due to aldehyde degradation (Heinzel, 1988). Formaldehyde vapour may be released by evaporating formalin solutions, by adding potassium permanganate to formalin or alternatively by heating, under controlled conditions, the polymer paraformaldehyde ($HO(CH_2O)_nH$), urea formaldehyde or melamine formaldehyde (Tulis, 1973). The activity of the vapour depends on aldehyde concentration, temperature and relative humidity (r.h.) (Section 18.2).

7.2.4 *Formaldehyde-releasing agents*

Noxythiolin (oxymethylenethiourea; Fig. 2.15a) is a bactericidal agent (Kingston, 1965; Wright & McAllister, 1967; Browne & Stoller, 1970) that apparently owes its antibacterial activity to the release of formaldehyde (Kingston, 1965; Pickard, 1972; cf. Gucklhorn, 1970):

$$CH_3{\cdot}NH{\cdot}CS{\cdot}NH{\cdot}CH_2OH \rightarrow CH_3{\cdot}NH{\cdot}CS{\cdot}NH_2H + CHO$$

Noxythiolin has been found to protect animals

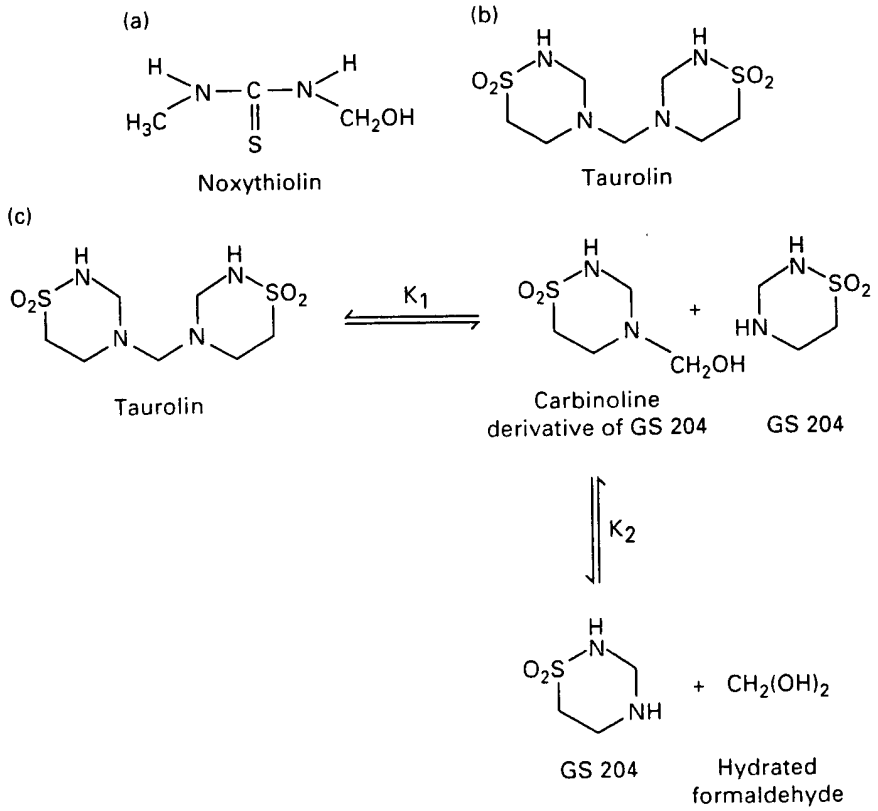


Fig. 2.15 (a) Noxythiolin; (b) taurolin; (c) postulated equilibrium of taurolin in aqueous solution (after Myers *et al.*, 1980).

from lethal doses of endotoxin (Wright & McAllister, 1967; Haler, 1974) and is claimed to be active against all bacteria, including those resistant to other types of antibacterial agents (Browne & Stoller, 1970).

Noxythiolin has been widely used both topically and in accessible body cavities, notably as an irrigation solution in the treatment of peritonitis (Pickard, 1972). Unfortunately, solutions are rather unstable (after preparation they should be stored at 10°C and used within 7 days). Commercially, noxythiolin is available as Noxyflex S and Noxyflex (Geistlich Ltd., Chester, UK), the latter containing amethocaine hydrochloride as well as noxythiolin. Solutions of Noxyflex (containing 1 or 2.5% noxythiolin) are employed where local discomfort is experienced.

More recently, the amino acid taurine has been selected as the starting-point in the design of a new antibacterial agent, taurolin (Fig. 2.15b), which is a condensate of two molecules of taurine and three molecules of formaldehyde. Taurolin (bis-(1,1-dioxoperhydro-1,2,4-thiazinyl-4)methane) is water-soluble and is stable in aqueous solution. It has a wide spectrum of antimicrobial activity *in vitro* and *in vivo* (Reeves & Schweitzer, 1973; Browne *et al.*, 1976, 1977, 1978).

Taurine is considered to act as a non-toxic formaldehyde carrier, donating methylol groups to bacterial protein and endotoxin (Browne *et al.*, 1976). According to these authors, taurine has a lower affinity for formaldehyde than bacterial protein, but a greater affinity than animal protein, the consequence of which is a selective lethal effect. Taurolin has been shown to protect experimental animals from the lethal effects of *E. coli* and *Bacteroides fragilis* endotoxin (Pfirrman & Leslie, 1979).

This viewpoint that the activity of taurolin results from a release of formaldehyde which is adsorbed by bacterial cells is, however, no longer tenable. When taurolin is dissolved in water (Myers *et al.*, 1980), an equilibrium is established (Fig. 2.15c) to release two molecules of the monomer (1,1-dioxoperhydro-1,2,4-thiadizine (GS 204)) and its carbinolamine derivative. The antibacterial activity of taurolin is considerably greater than that of free formaldehyde (Myers *et al.*, 1980; Allwood & Myers, 1981) and these authors thus concluded that the activity of taurolin was not due entirely to bacterial adsorption of free formaldehyde but also to a reaction with a masked (or latent) formaldehyde. Since GS 204 has only a low antibacterial effect, then the carbinolamine must obviously play an important role.

Clinically, the intraperitoneal administration of taurolin has been shown to bring about a significant reduction of morbidity in peritonitis (Browne *et al.*, 1978).

A third formaldehyde-releasing agent is hexamine (methenamine); hexamine itself is inactive but it breaks down by acid hydrolysis to release formaldehyde. It has been reviewed by Allwood & Myers (1981). Derivatives of hexamine are considered in Section 17.4 and other formaldehyde-releasing agents in Sections 17.2 (imidazole derivatives), 17.5 (triazines) and 17.6 (oxazolo-oxazoles). Table 2.18 should also be consulted, as well as Section 18.2 (which deals with release of gaseous formaldehyde) and Paulus (1976).

7.2.5 *Uses of formaldehyde*

Formaldehyde is employed as a disinfectant in both the liquid and gaseous states. Vapour-phase formaldehyde is used in the disinfection of sealed rooms; the vapour can be produced as described above, or alternatively an equal volume of industrial methylated spirits (IMS) can be added to formaldehyde and the mixture used as a spray. Other uses of formaldehyde vapour have been summarized by Russell (1976). These include the following: low-temperature steam plus formaldehyde vapour (LTSF) for the disinfection/sterilization of heat-sensitive medical materials (see also Chapter 19A); hospital bedding and blankets; and fumigation of poultry houses, of considerable importance in hatchery hygiene (Anon., 1970).

Aerobic spores exposed to liquid formaldehyde can be revived by a sublethal post-heat treatment (Spicher and Peters, 1976, 1981). Revival of LTSF-treated *Bacillus stearothermophilus* spores can also be accomplished by such means (Wright *et al.*, 1996), which casts considerable doubt on the efficacy of LTSF as a potential sterilizing process.

Formaldehyde in liquid form has been used as a viricidal agent in the production of certain types of viral vaccines, e.g. polio (inactivated) vaccine.

Formaldehyde solution has also been employed for the treatment of warts, as an antiseptic mouthwash, for the disinfection of membranes in dialysis equipment and as a preservative in hair shampoos. Formaldehyde-releasing agents were considered in Section 7.2.4. Formaldehyde and formaldehyde condensates have been reviewed in depth by Rossmore & Sondossi (1988).

7.3 Other aldehydes

Other aldehydes have been studied but results have sometimes been conflicting and they have thus been reinvestigated (Power & Russell, 1990). Sporidicin, used undiluted and containing 2% glutaraldehyde plus 7% phenol and 1.2% phenate, is slightly more active against spores than is 2% activated, alkaline glutaraldehyde. Gigasept, containing butan-1,4-dial, dimethoxytetrahydrofuran and formaldehyde, and used at 5% and 10% v/v dilutions, is considerably less active (Power & Russell, 1990).

o-Phthalaldehyde (Fig. 2.16) is a 'new' aldehyde. It is claimed to have potent bactericidal and viricidal activity (Alfa & Sitter, 1994) but information about its antimicrobial properties is currently based on limited experimentation. Further data about its activity and mechanisms of action are needed.

Glyoxal (2%) is weakly sporicidal, and butyraldehyde has no activity. It is essential that adequate procedures are employed to remove residual glutaraldehyde (and phenol/phenate, if present) or other aldehyde in determining survivor levels. This has not always been appreciated (Pepper, 1980; Leach, 1981; Isenberg, 1985).

The properties and uses of various aldehydes have been reviewed by Bartoli and Dusseau (1995).

8 Antimicrobial dyes

There are three groups of dyes which find application as antimicrobial agents: the acridines, the triphenylmethane group and the quinones.

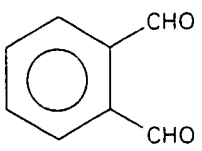


Fig. 2.16 *o*-Phthalaldehyde (OPA).

8.1 Acridines

8.1.1 Chemistry

The acridines (Fig. 2.17) are heterocyclic compounds that have proved to be of some value as antimicrobial agents. Acridine itself is feebly basic, but two of the five possible monoaminoacridines are strong bases, and these (3-aminoacridine and 9-aminoacridine) exist as the resonance hybrid of two canonical formulae. Both these monoacridines are well ionized as the cation at pH 7.3, and this has an important bearing on their antimicrobial activity (see below and Table 2.10). Further information on the chemistry of the acridines can be found in Albert's excellent book (Albert, 1966).

8.1.2 Antimicrobial activity

The acridines are of considerable interest because they illustrate how small changes in the chemical structure of the molecule cause significant changes in antibacterial activity. The most important limiting factor governing this activity is the degree of ionization, although this must be cationic in nature (Table 2.10). Acridine derivatives that form anions or zwitterions are only poorly antibacterial in comparison with those that form cations. In general terms, if the degree of ionization is less than 33% there is only feeble antibacterial activity, whereas above about 50% there is little further increase in activity (Albert, 1966).

In contrast to the triphenylmethane dyes (Section 8.2), the acridines do not display a selective action against Gram-positive organisms, nor are they inactivated by serum. Acridines compete with H^+ ions for anionic sites on the bacterial cell and are more effective at alkaline than acid pH (Browning *et al.*, 1919–20). They are relatively slow in their action and are not sporicidal (Foster & Russell, 1971). Resistance to the acridines develops as a result of mutation and indirect selection (Thornley & Yudkin, 1959a,b). Interestingly, acridines can eliminate ('cure') resistance in R^+ strains (see Watanabe, 1963, for an

Table 2.10 Dependence of antibacterial activity of acridines on cationic ionization (based on the work of Albert and his colleagues (see Albert, 1966)).

Substance	Predominant type (and percentage) of ionization at pH 3 and 37°C	Inhibitory activity
9-Aminoacridine	Cation (99%)	High
9-Aminoacridine-2-carboxylic acid	Zwitterion (99.8%)	Low
Acridine	Neutral molecule (99.7%)	Low
Acridine-9-carboxylic acid	Anion (99.3%)	Low

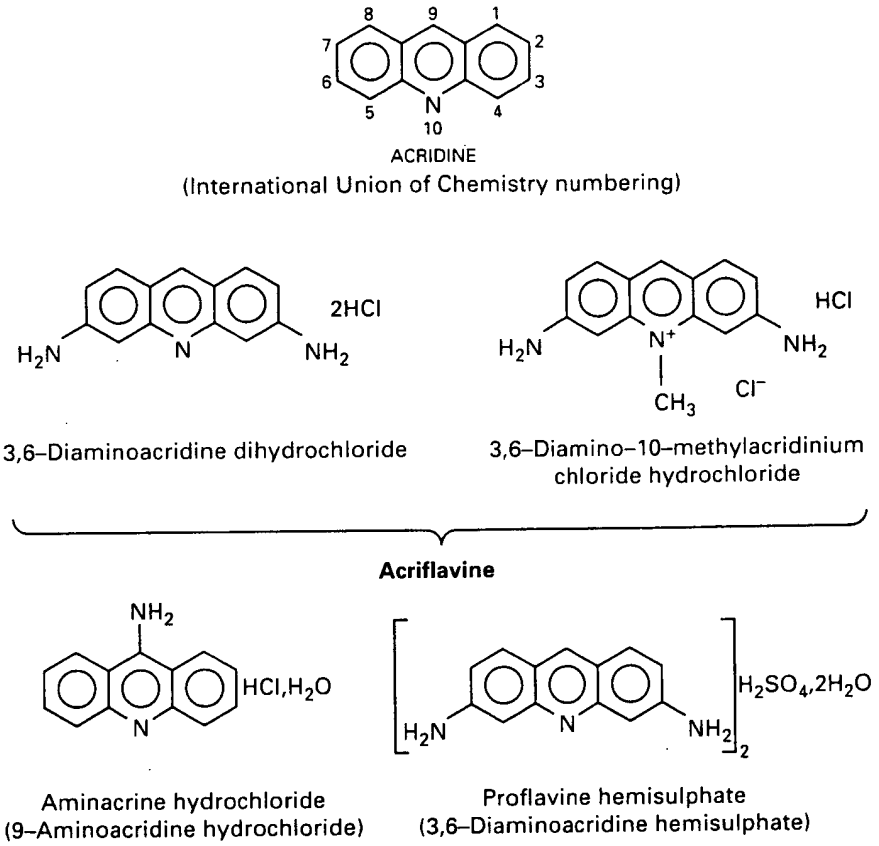


Fig. 2.17 Acridine compounds.

early review). Viljanen & Boratynski (1991) provide more recent information about plasmid curing.

The MRSA and methicillin-resistant *Staphylococcus epidermidis* (MRSE) strains are more resistance to acridines than are antibiotic-sensitive strains, although this resistance depends on the presence of *qac* genes, especially *qac*A or *qac*B Littlejohn *et al.*, 1992; Leelaporn *et al.*, 1994).

8.1.3 Uses

For many years, the acridines held a valuable place in medicine. However, with the advent of antibiotics and other chemotherapeutic agents, they are now used infrequently. Their major use has been the treatment of infected wounds. The first compound to be used medically was acriflavine (a mixture of 3,6-diaminoacridine hydrochloride and 3,6-diamino-10-methylacridinium hydrochloride, the former component being better known as proflavine). Proflavine hemisulphate and 9-aminoacridine (aminacrine) have found use in treating wounds; aminacrine is particularly useful as it is non-staining.

8.2 Triphenylmethane dyes

The most important members of this group are cyrstal violet, brilliant green and malachite green (Fig. 2.18). These were used as local antiseptics for application to wounds and burns, but were limited in being effective against Gram-positive bacteria (inhibitory concentrations 1 in 750 000 to 1 in 5 000 000) but much less so against Gram-negative organisms, and in suffering a serious decrease in activity in the presence of serum. Their selective activity against Gram-positive bacteria has a practical application in the formulation of selective media for diagnostic purposes, e.g. crystal violet lactose broth in water filtration-control work.

The activity of the triphenylmethane dyes is a property of the pseudobase, the formation which is established by equilibrium between the cation and the base; thus, both the ionization and the equilibrium constants will affect the activity (Albert, 1966). Antimicrobial potency depends on external pH, being more pronounced at alkaline values (Moats & Maddox, 1978).

For an extensive account of the antibacterial dyestuffs, see Browning (1964).

The MRSA and MRSE strains containing *qac* genes are more resistant to crystal violet than are plasmidless strains of *Staph. aureus* and *Staph. epidermidis*, respectively (Littlejohn *et al.*, 1992; Leelaporn *et al.*, 1994). This is believed to be the result of an efficient efflux system in the resistant strains (Paulsen *et al.*, 1996a,b). However, crystal violet finds little, if any, use nowadays as an antibacterial agent, and the clinical relevance of this finding thus remains uncertain (Russell & Chopra, 1996; Russell, 1997).

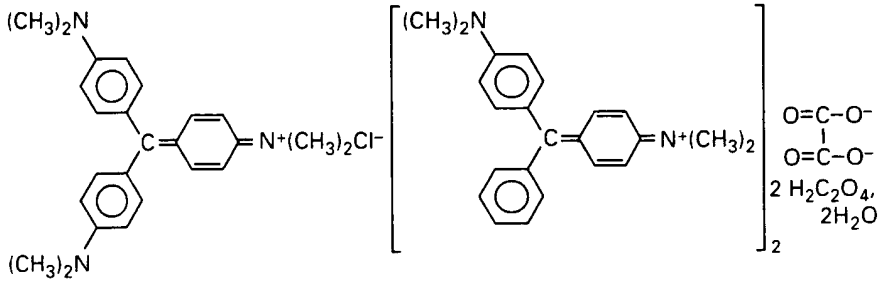
8.3 Quinones

Some members of this group of dyes are important agricultural fungicides. The quinones are natural dyes, which give colour to many forms of plant and animal life. Chemically (Fig. 2.19), they are diketocyclohexadienes; the simplest member is 1,4-benzoquinone. In terms of toxicity to bacteria, moulds and yeast, naphthaquinones are the most toxic, followed (in this order) by phenanthrenequinones, benzoquinones and anthraquinones.

Antimicrobial activity is increased by halogenation and two powerful agricultural fungicides are chloranil (tetrachloro-1,4-benzoquinone) and dichlone (2,3-dichloro-1,4-naphthaquinone); see D'Arcy (1971) and Owens (1969).

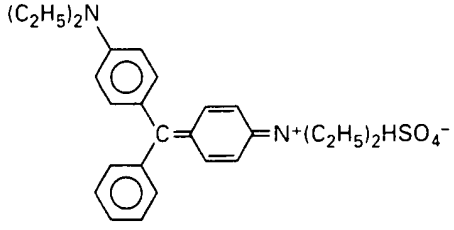
9 Halogens

The most important microbicidal halogens are iodine compounds, chlorine compounds and



Crystal violet
(methyl violet; gentian violet)

Malachite green



Brilliant green

Fig. 2.18 Triphenylmethane dyes.

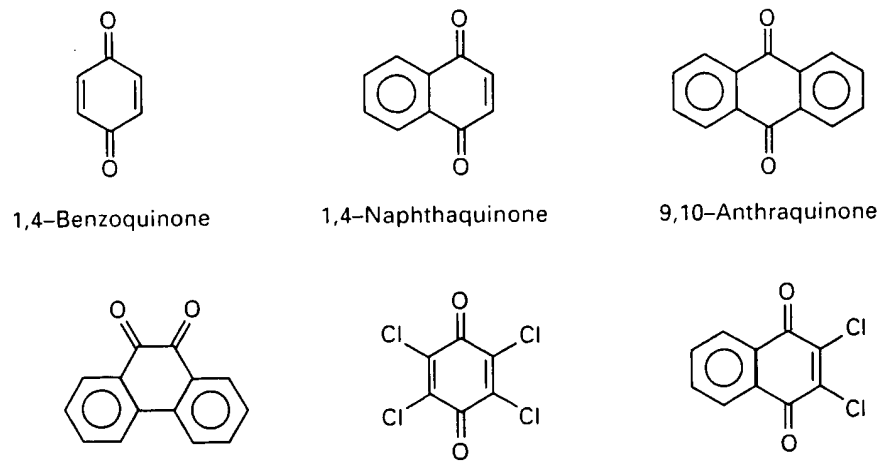


Fig. 2.19 Quinones.

bromine. Fluorine is far too toxic, irritant and corrosive for use as a disinfectant (Trueman, 1971), although, interestingly, fluoride ions have been shown to induce bacterial lysis (Lesher *et al.*, 1977). This section will deal predominantly with iodine, iodophors and chlorine-releasing compounds (those which are bactericidal by virtue of 'available chlorine'), but bromine, iodoform and (considered here for convenience) chloroform will be considered briefly.

9.1 Iodine compounds

9.1.1 Free iodine

Iodine was first employed in the treatment of wounds some 140 years ago and has been shown to be an efficient microbicidal agent with rapid lethal effects against bacteria and their spores, moulds, yeasts and viruses (Gershenfeld, 1956; Anon., 1965; Sykes, 1965; Russell, 1971b; Kelsey & Maurer, 1972). It is normally used in aqueous or alcoholic solution; it is only sparingly soluble in cold water but solutions can be made with potassium iodide. Iodine is less reactive chemically than chlorine, and is less affected by the presence of organic matter than is the latter; however, it must be added that, whereas the activity of high concentrations of iodine is little affected by organic matter, that of low concentrations is significantly lowered. The activity of iodine is greater at acid than at alkaline pH; see Table 2.11. Unfortunately, iodine solutions stain fabric and tend to be toxic.

9.1.2 Iodophors

Certain surface-active agents can solubilize iodine to form compounds (the iodophors) that retain the germicidal action but not the undesirable properties of iodine. The uses of the iodophors as detergent-sterilizers have been described by Blatt & Maloney (1961) and Davis (1962). The term iodophor itself means, literally, iodine-carrier. It must be noted that different concentrations of iodophors are used for antiseptic and disinfectant

Table 2.11 Effect of pH on the antimicrobial activity of iodine compounds (based on Trueman, 1971).

pH	Active form	Comment
Acid and neutral	I_2(diatomic iodine)	Highly bactericidal
	Hypo-iodous acid	Less bactericidal
Alkaline	Hypo-iodite ion	Even less bactericidal
	Iodate (IO_3^-), iodide (I^-) and tri-iodide (I_3^-) ions	All inactive

purposes, and that the lower concentrations employed in antisepsis are not claimed to be sporicidal (Favero, 1985, 1995).

Gershenfeld (1962) has shown that povidone-iodine is sporicidal, and Lowbury *et al.* (1964) found that povidone-iodine compressess reduced the numbers viable spores of *Bacillus globigii* on the skin by >99% in 1 h, suggesting that this iodophor had a part to play in removing transient sporing organisms from operation sites. The importance of povidone-iodine in preventing wound infection was re-emphasized as a result of the studies of Galland *et al.* (1977) and Lacey (1979).

The concentration of free iodine in aqueous or alcoholic iodine solutions is responsible for microbicidal activity. Likewise, the concentration of free iodine in an iodophor is responsible for its activity: this was proved by Allawala & Riegelman (1953), who made a log–log plot of the killing time against the amount of free iodine, and who showed that the 99% killing time against *B. cereus* spores was a function of the concentration of free iodine in the presence or absence of added surface-active agents.

In most iodophor preparations, the carrier is usually a non-ionic surfactant, in which the iodine is present as micellar aggregates. When an iodophor is diluted with water, dispersion of the micelles occurs and most (80–90%) of the iodine is slowly liberated. Dilution below the CMC of the non-ionic surface-active agent results in iodine being in simple aqueous solution. A paradoxical effect of dilution on the activity of povidone-iodine has been observed (Gottardi, 1985; Rackur, 1985). As the degree of dilution increases, then beyond a certain point bactericidal activity also increases. An explanation of this arises from consideration of physicochemical studies, which demonstrate that, starting from a 10% commercially available povidone-iodine solution, the concentration of non-complexed iodine (I_2) initially increases as dilution increases. This reaches a maximum value at about 0.1% and then falls. In contrast, the content of other iodine species, e.g. I^- and I_3^- decreases continuously. These properties affect the sporicidal activity of iodine solutions (Williams & Russell, 1991).

The iodophors, as stated above, are microbicidal, with activity over a wide pH range. The presence of a surface-active agent as carrier improves the wetting capacity. Iodophors may be used in the dairy industry (when employed in the cleansing of dairy plant it is important to keep the pH on the acid side to ensure adequate removal of milkstone) and for skin and wound disinfection. Iodophors, such as Betadine, in the form of alcoholic solutions are widely used in the USA for disinfection of hands for operation sites (see also Chapter 13). Pseudobacteraemia (false-positive blood cultures) has been found to result from the use of contaminated antiseptics. Craven *et al.* (1981) have described such an outbreak of pseudobacteraemia caused by a 10% povidone-iodine solution contaminated with Burkholderia (*Pseudomonas*) *cepacia*.

The properties, antimicrobial activity, mechanisms of action and uses of iodine and its compounds have been described by Rutala (1990), Favero & Bond (1991), Banner (1995), Favero (1995) and Bloomfield (1996). Information about the revival of iodine-treated spores of *B. subtilis* is provided by Williams & Russell (1992, 1993a, b,c).

9.1.3 *Iodoform*

When applied to tissues, iodoform (CHI_3) slowly releases elemental iodine. It thus has some weak antimicrobial activity. It is not often used in practice, and thus will not be considered further.

9.2 Chlorine compounds

9.2.1 *Chlorine-releasing compounds*

Until the development of chlorinated soda solution, surgical (Dakin's solution), in 1916, the commercial chlorine-releasing disinfectants then in use were not of constant composition and contained free alkali and sometimes free chlorine. The stability of free available chlorine in solution is dependent on a number of factors, especially the following (Dychdala, 1983):

1 Chlorine concentration.
2 pH of organic matter.
3 Presence the solution.
4 Light.

These factors are considered below.

The types of chlorine compounds that are most frequently used are the hypochlorites and *N*-chloro compounds (Trueman, 1971; Dychdala, 1983; Gardner & Peel, 1986, 1991; Favero & Bond, 1991; Bloomfield & Arthur, 1994; Banner, 1995; Favero, 1995; Bloomfield, 1996).

Hypochlorites. These have a wide antibacterial spectrum, although they are less active against spores than against non-sporulating bacteria and have been stated to be of low activity against mycobacteria (Anon., 1965; Croshaw, 1971). Recent studies have suggested that chlorine compounds are among the most potent sporicidal agents (Kelsey *et al.*, 1974; Coates & Death, 1978; Death & Coates, 1979; Coates & Hutchinson, 1994). The hypochlorites show activity against lipid and non-lipid viruses (Morris & Darlow, 1971; Favero, 1995; Bloomfield, 1996).

Two factors that can affect quite markedly their antimicrobial action are organic matter, since chlorine is a highly reactive chemical, and pH, the hypochlorites being more active at acid than at alkaline pH (Table 2.12). The former problem can, to some extent, be overcome by increasing the hypochlorite concentration, and it has been shown that the sporicidal activity of sodium hypochlorite (200 parts/10^6 available chlorine) can be potentiated by 1.5–4% sodium hydroxide, notwithstanding the above comment about pH (Russell, 1971b, 1982). The sporicidal activity can also be potentiated by low concentrations of ammonia (Weber & Levine, 1944) and in the presence of bromine (Farkas-Himsley, 1964); chlorine-resistant bacteria have been found to be unaffected by bromine but to be readily killed by chlorine–bromine solutions (Farkas-Himsley, 1964). Such mixtures could be of value in the disinfection of natural waters.

Organic chlorine compounds. *N*-chloro compounds, which contain the =N–Cl group, show microbicidal activity. Examples of such compounds, the chemical structures of which are shown in Fig. 2.20, are chloramine-T, dichloramine-T, halazone, halane, dichloroisocyanuric acid, sodium and potassium dichloroisocyanurates and trichloroisocyanuric acid. All appear to hydrolyse in water to produce an imino (=NH) group. Their action is claimed to be slower than that of the hypochlorites, although this can be increased under acidic conditions (Cousins & Allan, 1967). A series of imidazolidinone *N′*,*N′*-dihalamine disinfectants has been described (Williams *et al.*, 1987, 1988; Worley *et al.*, 1987). The dibromo compound (Fig. 2.20) was the most rapidly acting bactericide, particularly under halogen demand-free conditions, with the mixed bromo-chloro compound (Fig. 2.20) occupying an intermediate position. However, when stability of the compounds in the series was also taken into account, it was concluded that the mixed product was the most useful as an aqueous disinfectant solution.

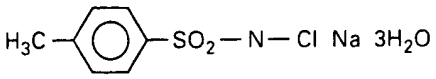
Coates (1985) found that solutions of sodium hypochlorite (NaOCl) and sodium dichloroisocyanurate (NaDCC) containing the same levels of available chlorine had similar bactericidal activity despite significant differences in their pH. Solutions of NaDCC are less susceptible than NaOCl

Table 2.12 Factors influencing activity of hypochlorites.

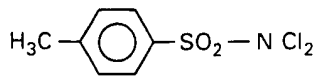
Factor	Result
pH	Activity decreased by increasing pH (see text and use of NaOH also)
Concentration of hypochlorite (pH constant)	Activity depends on concentration of available chlorine
Organic matter	Antimicrobial activity reduced considerably
Other agents	Potentiation may be achieved by 1 addition of ammonia 2 1.5–4% sodium hydroxide* 3 addition of small amounts of bromide†

*Cousins & Allan (1967).

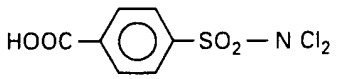
†In the presence of bromide, hypochlorite also has an enhanced effect in bleaching cellulosic fibres.



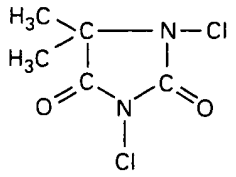
Chloramine T
(sodium-*p*-toluene-sulphonchloramide)



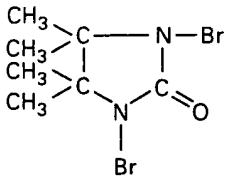
Dichloramine T
(*p*-toluene-sulphondichloramide)



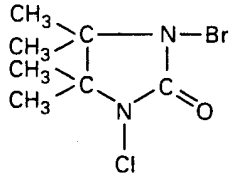
Halazone
(*p*-sulphondichloramide benzoic acid)



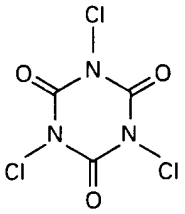
Halane



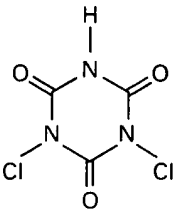
1,3-dibromo-4,4,5,5-
tetramethyl-2-imidazoldinone



1-bromo-3-chloro-4,4,5,5-
tetramethyl-2-imidazolidinone



Trichloroisocyanuric acid



Dichloroisocyanuric acid

Fig. 2.20 Organic chlorine compounds.

to inactivation by organic matter (Bloomfield & Miles, 1979a,b; Bloomfield & Uso, 1985; Coates, 1985, 1988).

Uses of chlorine-releasing compounds. Chlorinated soda solution (Dakin's solution), which contains 0.5–0.55% (5000–5500 parts/10^6) available chlorine, and chlorinated lime and boric acid solution (Eusol), which contains 0.25% (2500 parts/10^6) available chlorine, are chlorine disinfectants that contain chlorinated lime and boric acid. Dakin's solution is used as a wound disinfectant or, when appropriately diluted, as an irrigation solution for bladder and vaginal infections. Eusol is used as a wound disinfectant, but Morgan (1989) has suggested that chlorinated solutions delay wound healing.

Chlorine gas has been employed to disinfect public water-supplies. Sodium hypochlorite is normally used for the disinfection of swimming-pools.

Blood spillages containing HIV or HBV can be disinfected with NaOCl solutions containing 10 000 parts/10^6 available chlorine (Working Party, 1985). Added directly to the spillage as powder or granules, NaDCC is also effective, may give a larger margin of safety because a higher concentration of available chlorine is achieved and is also less susceptible to inactivation by organic matter, as pointed out above (Coates, 1988). Furthermore, only a very short contact time (2–3 min) is necessary before the spill can be removed safely (Coates & Wilson, 1989). Chlorine-releasing powder formulations with high available chlorine concentrations are particularly useful for this purpose (Bloomfield & Miller, 1989; Bloomfield *et al.*, 1990).

Chlorine dioxide, an alternative to sodium

hypochlorite, is more active at alkaline pH and in the presence of organic matter and more environmentally satisfactory (BS 7152, 1991).

Additional information. Chlorine-releasing agents continue to be widely studied. Their sporicidal activity has been described by Te Giffel *et al.* (1996) and Coates (1996), their antiviral efficacy by Bellamy (1995), van Bueren (1995), Bond (1995) and Hernandez *et al.* (1996, 1997) and their usefulness in dental practice by Molinari (1995), Cottone & Molinari (1996) and Gurevich *et al.* (1996).

9.2.2 *Chloroform*

Chloroform ($CHCl_3$) has been used as a preservative in many pharmaceutical products intended for internal use, for more than a century. In recent years, with the object of minimizing microbial contamination, this use has been extended. Various authors, notably Westwood & Pin-Lim (1972) and Lynch *et al.* (1977), have shown chloroform to be a bactericidal agent, although it is not sporicidal and its high volatility means that a fall in concentration could result in microbial growth. For details of its antibacterial activity in aqueous solutions and in mixtures containing insoluble powders and the losses, through volatilization, under 'in-use' conditions, the paper by Lynch *et al.* (1977) should certainly be consulted.

The present position is that chloroform may be used in oral pharmaceutical products at concentrations of no greater than 0.5%; in cosmetic products its use (at a maximum concentration of 4%) will be restricted to toothpaste. It is totally banned in the USA. It is noteworthy that, in an article on the preservation of cosmetics and toiletries, Hill (1995) describes those cosmetic preservatives allowed in the European Union (EU): chloroform is not included in this list.

9.3 Bromine

The antimicrobial activity of bromine was first observed in the 1930s, but it was not until the 1960s that it was used commercially in water disinfection. The most commonly used oxidizing biocide in recirculating waters is chlorine, but bromine has been put forward as an alternative (Elsmore, 1993).

Elemental bromine is not itself employed commercially. The two available methods (Elsmore, 1995) are: (i) activated bromide produced by reacting sodium bromide with a strong oxidizing agent, such as sodium hypochlorite or gaseous chlorine; and (ii) organic bromine-releasing agents, such as *N*-bromo-*N*-chlorodimethylhydantoin (BCDMH; Fig. 2.21a). When BCDMH hydrolyses in water, it liberates the biocidal agents hypobromous acid (HOBr) and hypochlorous acid (HOCl), together with the carrier, dimethylhydantoin (DMH; Fig. 2.21b).

Both HOBr and HOCl would appear to contribute towards the overall germicidal activity of BCDMH. However, Elsmore (1993, 1995) has pointed out that the primary agent present in water is HOBr. Hypochlorous acid is used up in regenerating 'spent bromine' produced when HOBr reacts with organic materials and microorganisms:

$$HOCl + Br^- \rightarrow HOBr + Cl^-$$

Bromine is claimed to have a greater bactericidal activity than chlorine. It is effective against *Legionella pneumophila* in the laboratory and in field studies (McCoy & Wireman, 1989). The pK_a for HOBr (8.69) is higher than that for HOCl (7.48) and thus, at the normal alkaline pH values found in cooling towers, there is a significantly higher amount of active biocide present with HOBr than with HOCl.

10 Quinoline and isoquinoline derivatives

There are three main groups of derivatives:

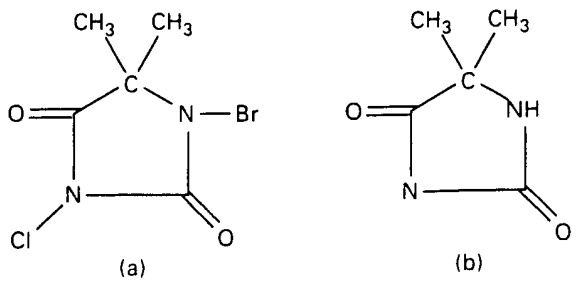
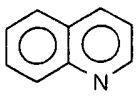
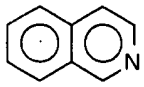


Fig. 2.21 (a) Bromochlorodimethylhydantoin (BCDMH); (b) dimethylhydantoin (DMH).

(a)

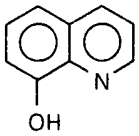


Quinoline

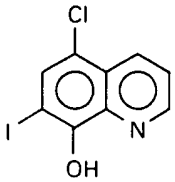


Isoquinoline

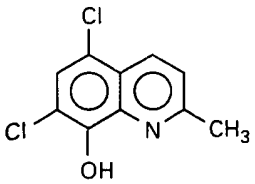
(b)



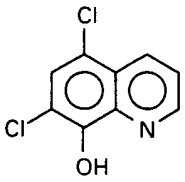
8-Hydroxyquinoline



Clioquinol
(5-chloro-8-hydroxy-7-iodoquinoline)



Chloroquinaldol
(5,7-dichloro-8-hydroxy-2-methyl-quinoline)



Halquinol
(chlorinated 8-hydroxyquinoline, 65% of which is 5,7-dichloro-8-hydroxyquinoline, shown above)

(c)

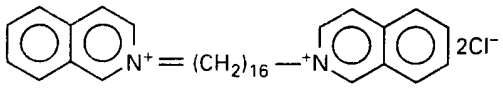
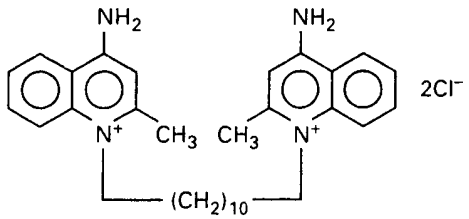


Fig. 2.22 (a) Structures of quinoline and isoquinoline; (b) 8-hydroxyquinoline derivatives with antimicrobial properties; (c) hedaquinium chloride.

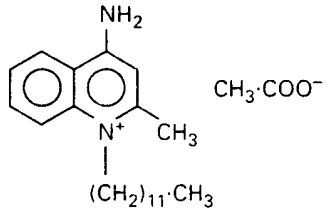
8-hydroxyquinoline derivatives, 4-aminoquinaldinium derivatives and isoquinoline derivatives. They are described in Figs. 2.22 and 2.23.

10.1 8-Hydroxyquinoline derivatives

8-Hydroxyquinoline (oxine) possesses antibacterial activity against Gram-positive bacteria, but much less against Gram-negative organisms. It also has antifungal activity, although this occurs at a slower rate. Other useful compounds are depicted in Fig. 2.22a). Like oxine, clioquinol, chlorquinandol and halquinol have very low water solubilities, and are generally employed as applications to the skin. An interesting feature of their activity (discussed in more detail in Chapter 9) is the fact that they are chelating agents, which are active only in the presence of certain metal ions.



Dequalinium chloride



Laurolinium acetate

Fig. 2.23 4-Aminoquinaldinium derivatives with antimicrobial properties.

10.2 4-Aminoquinaldinium derivatives

These are QACs (see Fig. 2.23), which also fall into this grouping. The most important members are laurolinium acetate and dequalinium chloride (a bis-QAC). Both compounds possess anti-

bacterial activity, especially against Gram-positive bacteria (Collier *et al.*, 1959; Cox & D'Arcy, 1962), as well as significant activity against many species of yeasts and fungi (Frier, 1971; D'Arcy, 1971). Their activity is decreased in the presence of lecithin; serum decreases the effectiveness of laurolinium but not of dequalinium. Dequalinium chloride is used as lozenges or paint in the treatment of infections of the mouth and throat. Laurolinium has been used as a preoperative skin disinfectant, although this was never widely adopted.

10.3 Isoquinoline derivatives

The most important isoquinoline derivative is hedaquinium chloride (Fig. 2.22c), another bis-quaternary salt. This possesses antibacterial and antifungal activity (Collier *et al.*, 1959; D'Arcy, 1971), and is regarded as one of the most active antifungal QAC agents (D'Arcy, 1971).

11 Alcohols

Several alcohols have been shown to possess antimicrobial properties. Generally, the alcohols have rapid bactericidal activity (Morton, 1950), including acid-fast bacilli, but are not sporicidal; they have low activity against some viruses, but are viricidal towards others. Their chemical structures are shown in Fig. 2.24.

11.1 Ethyl alcohol (ethanol)

Ethanol is rapidly lethal to non-sporulating bacteria and destroys mycobacteria (Croshaw, 1971) but is ineffective at all concentrations against bacterial spores (Russell, 1971b). The presence of water is essential for its activity, but concentrations below 30% have little action. Activity, in fact, drops sharply below 50% (Rutala, 1990).

The most effective concentration is about 60–70% (Price, 1950; see also Croshaw, 1977; Morton, 1977; Scott & Gorman, 1987). Solutions of iodine or chlorhexidine in 70% alcohol may be employed for the preoperative disinfection of the skin. Ethanol is the alcohol of choice in cosmetic products because of its relative lack of odour and irritation (Bandelin, 1977).

Some variable results have been reported about the effects of ethanol on HIV. Tjøtta *et al.* (1991) showed that 70% ethanol in the presence of 2.5% serum produced a 3-log/ml reduction in virus titre after a 10-min contact period, as determined by plaque assay or immunofluorescence. In contrast, using a $TCID_{50}$ assay, Resnick *et al.* (1986) found that 70% alcohol after 1min and in the presence of 50% plasma yielded a 7-log reduction in $TCID_{50}$/ml, again in a suspension test. Van Bueren *et al.* (1994) also described a rapid inactivation of HIV-1 in suspension, irrespective of the protein load. The rate of inactivation decreased when high protein levels were present when a carrier test was employed. A notable feature of the experiments carried out by van Buesen *et al.* (1994) was the care taken to ensure that residual alcohol was neutralized to prevent toxicity to the cell line employed in detecting uninactivated virus.

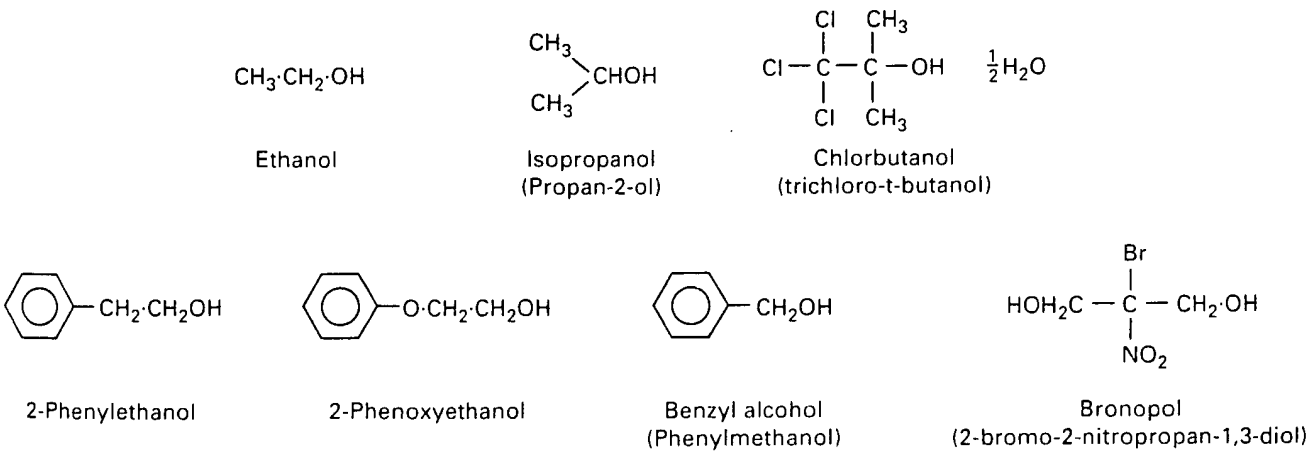


Fig. 2.24 Alcohols.

The non-enveloped poliovirus is more resistant to biocides in general than the herpesvirus, and ethanol caused no inactivation of poliovirus in a suspension test (Tyles *et al.*, 1990).

11.2 Methyl alcohol (methanol)

Methyl alcohol has poor antibacterial activity and is not sporicidal (Russell, 1971b; Bandelin, 1977; Coates & Death, 1978; Death & Coates, 1979). Furthermore, it is potentially toxic, and is thus little used. However, freshly prepared mixtures of alcohols (especially methanol) and sodium hypochlorite are highly sporicidal (Coates & Death, 1978). Although it was then considered that methanol was potentiating the activity of hypochlorites, it is, in fact, more likely that hypochlorites, by virtue of their effects on the outer spore layers (Bloomfield and Arthur, 1994), are aiding the penetration of methanol into the spore.

11.3 Isopropyl alcohol (isopropanol)

Isopropyl and *n*-propyl alcohols are more effective bactericides than ethanol (Anon., 1965; Kelsey & Maurer, 1972), but are not sporicidal. They are miscible with water in all proportions, but isopropanol has a less objectionable odour than *n*-propanol and is considered as a suitable alternative to ethanol in various cosmetic products, either as a solvent or as a preservative (Bandelin, 1977; Hill, 1995).

Isopropanol has viricidal activity, but not towards 'hydrophilic' (non-lipid-enveloped) viruses (Rutala, 1990). Van Bueren *et al.* (1994) have demonstrated inactivation of HIV type 1 by isopropanol. For further information, the papers by Tyler *et al.* (1990) and Sattar & Springthorpe (1991) should be consulted.

11.4 Benzyl alcohol

In addition to having antimicrobial properties, benzyl alcohol is a weak local anaesthetic. It has activity against Gram-positive and Gram-negative bacteria and against moulds (D'Arcy, 1971).

Benzyl alcohol is incompatable with oxidizing agents and is inactivated by non-ionic surfactants; it is stable to autoclaving and is normally used at a concentration of 1% v/v (Denyer & Wallhäusser, 1990).

11.5 Phenylethanol (phenylethyl alcohol)

Phenylethyl alcohol is an antimicrobial agent with selective activity against various bacteria (especially Gram-negative (Lilley & Brewer, 1953) and which has been recommended for use as a preservative in ophthalmic solutions, often in conjunction with another microbicide. Because of its higher activity against Gram-negative bacteria, phenylethyl alcohol may be incorporated into culture media for isolating Gram-positive bacteria from mixed flora, e.g. phenylethyl alcohol agar.

Phenylethanol is commonly used at a concentration of 0.3–0.5% v/v; it shows poor stability with oxidants and is partially inactivated by non-ionic surfactants (Denyer & Wallhäusser, 1990).

11.6 Bronopol

Bronopol, 2-bromo-2-nitropropan-1,3-diol, is an aliphatic halogenonitro compound with antibacterial and antifungal activity, although bacterial spores are unaffected. It is effective against *P. aeruginosa*. Its activity is reduced somewhat by 10% serum and to a greater extent by sulphydryl compounds, but is unaffected by 1% polysorbate or 0.1% lecithin. It has a half-life of about 96 days at pH and 25°C (Toler, 1985).

Bronopol is most stable under acid conditions; the initial decomposition appears to involve the liberation of formaldehyde and the formulation of bromonitroethanol (Fig. 2.25a). A second-order

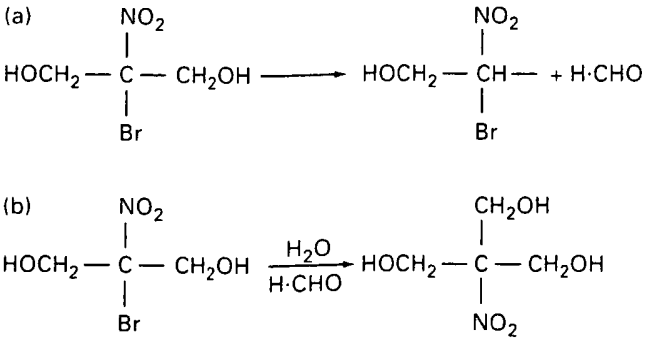


Fig. 2.25 (a) Initial process in the decomposition of bronopol; (b) second-order reaction involving bronopol and formaldehyde.

reaction involving bronopol and formaldehyde occurs simultaneously to produce 2-hydroxymethyl-2-nitro-1,3-propanediol (Fig. 2.25b), which itself decomposes with the loss of formaldehyde.

Details of the microbiological activity, chemical stability, toxicology and uses of bronopol are documented by Bryce *et al.* (1978), Croshaw & Holland (1984), Toler (1985) and Rossmore & Sondossi (1988). Denyer & Wallhäusser (1990) have provided useful information about bronopol, the typical in-use concentration of which is 0.01–0.1% w/v. Sulphydryl compounds act as appropriate neutralizers in preservative efficacy tests.

11.7 Phenoxyethanol (phenoxetol)

The antimicrobial activity of phenoxyethanol and other preservatives has been reviewed by Gucklhorn (1970, 1971). Phenoxyethanol was shown by Berry (1944) to possess significant activity against *P. aeruginosa*, but it has less activity against other Gram-negative organisms or against Gram-positive bacteria. Phenoxyethanol is stable to autoclaving and is compatible with anionic and cationic surfactants, but it shows reduced activity in the presence of polysorbate 80. It is used as a preservative, typical concentration 1% (Denyer & Wallhäusser, 1990).

11.8 Chlorbutanol (chlorbutol)

Chlorbutol is an antibacterial and antifungal agent. It has been used, at a concentration of 0.5% w/v, as a bactericide in injections. One drawback to its employment is its instability, since at acid pH it decomposes at the high temperature used in sterilization processes into hydrochloric acid, and at alkaline pH it is unstable at room temperature.

Chlorbutanol is incompatible with some non-ionic surfactants. Its typical in-use concentration as a pharmaceutical preservative is 0.3–0.5% w/v (Denyer & Wallhäusser, 1990).

11.9 2,4-Dichlorobenzyl alcohol

This substance is a white powder, soluble in water to 1% and readily soluble in alcohols. Its ionization is negligible for all practical purposes and it is thus active over a wide pH range. It has a broad spectrum of activity, but both pseudomonads and *S. aureus* show some resistance to it (Toler, 1985).

12 Peroxygens

12.1 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a familiar household antiseptic. It was discovered in 1818 and was early recognized as possessing antibacterial properties. These were extensively investigated in 1893 by Traugott.

Hydrogen peroxide is available as a solution designated as 20- or 10-volume, a means of indicating its strength by describing the volume (20 or 10, respectively) of oxygen evolved from 1 volume of the peroxide solution. Strengths for industrial use of 35, 50 or 90% are available. Hydrogen peroxide solutions are unstable, and benzoic acid or another suitable substance is added as a stabilizer.

Hydrogen peroxide solutions possess disinfectant, antiseptic and deodorant properties. When in contact with living tissue and many metals they decompose, evolving oxygen. Hydrogen peroxide is bactericidal and sporicidal (Russell, 1982, 1990a,b, 1991a,b; Baldry, 1983; Baldry & Fraser, 1988) and is believed to act as a generator of free hydroxyl radicals, which can cause DNA strand breakage. It is an oxidizing agent and reacts with oxidizable material, for example alkali nitrites used in anticorrosion solutions. It is environmentally friendly because its decomposition products are oxygen and water (Miller, 1996).

Hydrogen peroxide has been used in aseptic packaging technology and for disinfecting contact lenses.

Microbial inactivation is more rapid with liquid peroxide than with vapour generated from that liquid acting at the same temperature (Sintim-Damoa, 1993). However, the vapour can be used for the purposes of sterilization, where, at a concentration of 1–5 mg/l, it generally shows good penetration.

Attention has recently been devoted to developing a plasma-activated peroxide vapour process, in which radio waves produce the plasma. This is believed to be microbicidal by virtue of the

hydroxyl ions and other free radicals that are generated (Groschel, 1995; Lever & Sutton, 1996).

The use of hydrogen peroxide as a contact-lens disinfectant has been reviewed (Miller, 1996).

12.2 Peracetic acid

Peracetic acid, $CH_3{\cdot}COOOH$, was introduced as an antibacterial agent in 1955. It is available commercially as a 15% aqueous solution, in which an equilibrium exists between peracetic acid and its decomposition products acetic acid ($CH_3{\cdot}COOH$) and hydrogen peroxide.

Peracetic acid solution has a broad spectrum of activity, including bacteria and their spores, moulds, yeasts, algae and viruses. It finds extensive use in the food industry and for disinfecting sewage sludge. It is a powerful oxidizing agent and in certain situations can be corrosive. The great advantage of peracetic acid is that its final decomposition products, oxygen and water, are innocuous.

More comprehensive data on peracetic acid are provided by Baldry (1983), Fraser (1986), Baldry & Fraser (1988), Coates (1996) and Russell & Chopra (1996).

13 Chelating agents

This section will deal briefly with chelating agents based on EDTA. Ethylenediamine tetraacetic acid has been the subject of intensive investigation for many years, and its antibacterial activity has been reviewed by Russell (1971a), Leive (1974) and Wilkinson (1975). The chemical nature of its complexation with metals has been well considered by West (1969).

The chemical structures of EDTA, ethylenedioxybis(ethyliminodi(acetic acid)) (EGTA), *N*-hydroxyethylethylenediamine-*NN'N'*-triacetic acid (HDTA), *trans*-1,2-diaminocyclohexane-*NNN'N'*-tetra-acetic acid (CDTA), iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) are provided in Fig. 2.26. Table 2.13 lists their chelating and antibacterial activities.

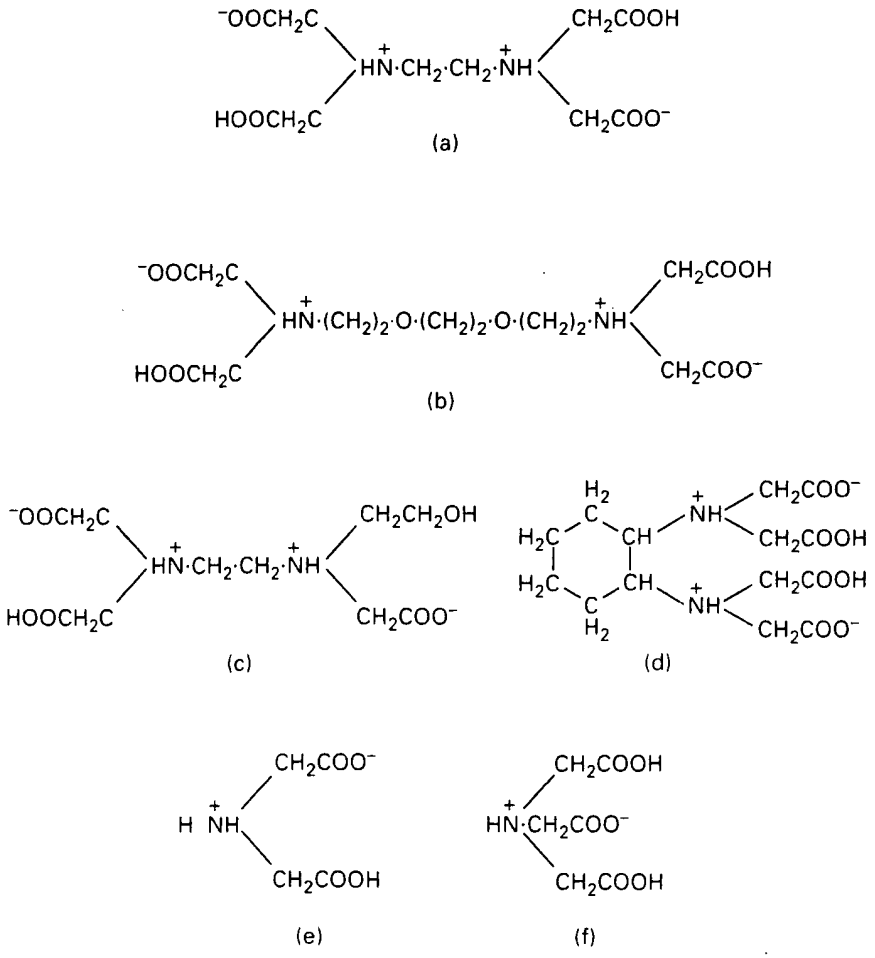


Fig. 2.26 Chelating agents. (a) Ethylenediamine tetraacetic acid (EDTA); (b) ethylenedioxybis (ethyliminodi(acetic acid)) (EGTA); (c) *N*-hydroxyethylenediamine-*NN'N'*-triacetic acid (HDTA); (d) *trans*-1,2-diaminocyclohexane-*NNN'N'*-tetraacetic acid (CDTA); (c) iminodiacetic acid (IDA); (f) nitrilotriacetic acid (NTA).

Table 2.13 Properties of chelating agents.

Property	EDTA	EGTA	HDTA	CDTA	IDA	NTA
Log stability constant*						
Ba	7.76	8.41	5.54	7.99	1.67	4.82
Ca	10.70	11.0	8.0	12.5	2.59	6.41
Mg	8.69	5.21	5.2	10.32	2.94	5.41
Zn	16.26	14.5	14.5	18.67	7.03	10.45
Antibacterial activity†						
Alone	Good	Good	Good	Low	Low	
As a potentiating agent for disinfectants	Yes		Yes	Yes	Somewhat	Somewhat

*Abstracted from the information supplied by West (1969).
†Based on the activity against *P. aeruginosa* described by Roberts *et al.* (1970) and Haque & Russell (1974a,b).

13.1 Ethylendiamine tetraacetic acid

In medicine, EDTA is commonly employed as the sodium or calcium–sodium salts. Sodium calcium edetate is used in the treatment of chronic lead poisoning, and the sodium salts are used clinically to chelate calcium ions, thereby decreasing serum calcium. Also EDTA is used as a stabilizing agent in certain injections and eye-drop preparations (Russell *et al.*, 1967).

The most important early findings, in a microbiological context, were made by Repaske (1956, 1958), who showed that certain Gram-negative bacteria became sensitive to the enzyme lysozyme in the presence of EDTA in tris buffer and that EDTA alone induced lysis of *P. aeruginosa*. The importance of tris itself has also been recognized (Leive & Kollin, 1967; Neu, 1969), since it appears to affect the permeability of the wall of various Gram-negative bacteria, as well as the nucleotide pool and RNA, which may be degraded. A lysozyme–tris–EDTA system in the presence of sucrose is a standard technique for producing spheroplasts/protoplasts in Gram-negative bacteria (McQuillen, 1960). During this conversion, several enzymes are released into the surrounding medium. A technique known as 'cold shock', which involves treating *E. coli* with EDTA + tris in hypertonic sucrose, followed by rapid dispersion in cold magnesium chloride—thus producing a sudden osmotic shift—again results in the release of enzymes, but without destroying the viability of the cells.

In the context of disinfection, EDTA is most important in that it will potentiate the activity of many antibacterial agents against many types of Gram-negative but not Gram-positive bacteria. This was clearly shown by Gray & Wilkinson (1965) and has since been confirmed and extended (Russell, 1971a; Wilkinson, 1975). An interesting offshoot was the development of Dettol Chelate, which consists of chloroxylenol and EDTA in a suitable formulation; unlike chloroxylenol alone, this new product has significant activity against *P. aeruginosa* strains (Russell & Furr, 1977). Ethylemediamine tetraacetic acid induces a non-specific increase in the permeability of the outer envelope of Gram-negative cells (Leive, 1974), thereby allowing more penetration of non-related agents. Ayres *et al.* (1993) reported on the permeabilizing activity of EDTA and other agents against *P. aeruginosa* in a rapid test method, the principle of which was the rapid lysis induced in this organism on exposure to the presumed permeabilizing agent plus lysozyme, an enzyme normally excluded in whole cells from its peptidoglycan target.

The mechanism of action of EDTA is dealt with in Chapter 9.

13.2 Other chelating agents

Chelating agents other than EDTA are described chemically in Fig. 2.26, and some of their properties (based in part on the excellent book of West, 1969) are listed in Table 2.13. While EGTA forms a stronger complex with Ca than does EDTA, for most other metals, except Ba and Hg, it is a weaker complexing agent than EDTA.

Notably, there is a divergency of 5.79 log K units between the stability constants of the Ca and Mg complexes with EGTA (West, 1969). Compared with EDTA, CDTA has superior complexing powers and it is better than all the other chelating agents listed in complexing Mg^{2+} ions. From a microbiological point of view, CDTA was found by Roberts *et al.* (1970) and Haque & Russell (1974a,b) to be the most toxic compound to *P. aeruginosa* and other Gram-negative bacteria in terms of leakage, lysis and loss of viability and in extracting metal ions from isolated cell envelopes (Haque & Russell, 1976).

The chelating agent HDTA corresponds to EDTA, one acetic acid of the latter molecule being replaced by a hydroxyethyl group. Its complexes are invariably less stable than those of EDTA. In a microbiological context, HDTA was found (Haque & Russell, 1976) to be rather less effective than EDTA.

Iminodiacetic acid forms weak complexes with most metal ions, whereas NTA is more reactive. Both have little activity against *P. aeruginosa*, although both, to some extent, potentiate the activity of other agents (disinfectants) against this organism.

14 Permeabilizers

Permeabilizers (permeabilizing agents) are chemicals that increase bacterial permeability to biocides (Vaara, 1992). Such chemicals include chelating agents, described above in Section 13, polycations, lactoferrin, transferrin and the salts of certain acids.

14.1 Polycations

Polycations such as poly-L-lysine ($lysine_{20}$; PLL) induce lipopolysaccharide (LPS) release from the outer membrane of Gram-negative bacteria. Organisms treated with PLL show greatly increased sensitivity to hydrophobic antibiotics (Vaara & Vaara, 1983a,b; Viljanen, 1987) but responses to biocides do not appear to have been studied.

14.2 Lactoferrin

Lactoferrin is an iron-binding protein that acts as a chelator, inducing partial LPS loss from the outer membrane of Gram-negative bacteria (Ellison *et al.*, 1988).

Lactoferricin B is a peptide produced by gastric peptic digestion of bovine lactoferrin. It is a much more potent agent than lactoferrin, binds rapidly to the bacterial cell surface and damages the outer membrane but has reduced activity in the presence of divalent cations (Jones *et al.*, 1994).

14.3 Transferrin

This iron-binding protein is believed to have a similar effect to lactoferrin (Ellison *et al.*, 1988). All are worthy of further studies as potentially important permeabilizers.

14.4 Citric and other acids

Used at alkaline pH, citric, gluconic and malic acids all act as permeabilizers (Ayres *et al.*, 1993). They perform as chelating agents and activity is reduced in the presence of divalent cations.

15 Heavy-metal derivatives

The historical introduction (Chapter 1) has already described the early use of high concentrations of salt employed empirically in the salting process as a preservative for meat, and the use of copper and silver vessels to prevent water from becoming fouled by microbial growth. Salting is still used in some parts of the world as a meat preservative and salts of heavy metals, especially silver, mercury, copper and, more recently, organotin, are still used as antimicrobial agents. The metal derivatives of copper, mercury, silver and tin, which find use as antiseptics and preservatives, will be discussed in this chapter. Kushner (1971) has reviewed the action of solutes other than heavy metal derivatives on microorganisms.

In addition to possessing antimicrobial activity in their own right, many metal ions are necessary for the activity of other drugs. A typical example is 8-hydroxyquinoline (Section 10.1), which needs Fe^{2+} for activity. The interesting relationship between antimicrobial compounds and metal cations has been reviewed by Weinberg (1957).

15.1 Copper compounds

Although the pharmacopoeias list a number of recipes containing copper salts (sulphate, actetate, citrate) as ingredients of antiseptic astringent lotions, the main antimicrobial use of copper derivatives is in algicides and fungicides. The copper(II) ion Cu^{2+} is pre-eminently an algicidal ion and at a final concentration of 0.5–2.9 µg/ml, as copper sulphate, it has been used to keep swimming-pools free from algae. Copper is thought to act by the poisoning effect of the copper(II) ion on thiol enzymes and possibly other thiol groups in microbial cells.

Copper sulphate and copper sulphate mixed with lime, Bordeaux mixture, introduced in 1885, are used as fungicides in plant protection. The latter formulation proved especially efficacious, as it formed a slow-release copper complex which was not easily washed from foliage. It was said to be first used as a deterrent to human predators of the grape crop and its antifungal properties emerged later. Copper metal, in powder form, finds an interesting application as an additive to cements and concretes. Its function is to inhibit microbial attack on the ingredients of these artificial products. The uses of copper metal here, and as vessels for drinking-water in the ancient world, illustrate a phenomenon which has been called the oligodynamic action of metals (Langwell, 1932). Metals are slightly soluble in water and in the case of copper, and also silver (q.v.), a sufficient concentration of ions in solution is achieved to inhibit microbial growth. Copper complexes, e.g. copper naphthenate and copper-7-hydroxyquinolate, have been particularly successful in the preservation of cotton fabrics. Wood, paper and paint have also been successfully preserved with copper compounds. As the preservation of paints, timber, etc. will be dealt with elsewhere in this volume (see Chapter 18), this chapter will merely summarize, by means of Table 2.14, some copper compounds and their application.

Table 2.14 Copper compounds used as preservatives and some examples of their application.

Compound	Example(s) of application
Copper metal	Concrete
Copper sulphate	Wood, water
Cuprammonium hydroxide	
Cuprammonium carbonate	
Cuprammonium fluoride	Fabrics, especially cellulosics
Copper chromate	
Copper borate	
Cuprous oxide	Paints, dark shades
Copper acetoarsenite	Paints, green shades
Copper oleate	
Copper stearate	Fabrics
Copper formate	
Copper naphthenate	Wood, fabric
Copper-8-hydroxyquinolate	Paint, papers
Copper phenylsalicylate	
Copper pentachlorphenate	Fabric

15.2 Silver compounds

Silver and its compounds have found a place in antimicrobial application from ancient times to the present day (Weber & Rutala, 1995). Apart from the use of silver vessels to maintain water in a potable state, the first systematic use of a silver compound in medicine was its use in the prophylaxis of ophthalmia neonatorum by the installation of silver nitrate solution into the eyes of newborn infants. Silver compounds have been used in recent years in the prevention of infection in burns, but are not very effective in treatment. An organism frequently associated with such infections is *P. aeruginosa*, and Brown & Anderson (1968) have discussed the effectiveness of Ag^+ in the killing of this organism. Among the Enterobacteriaceae, plasmids may carry genes specifying resistance to antibiotics and to metals. Plasmid-mediated resistance to silver salts is of particular importance in the hospital environment, because silver nitrate and silver sulphadiazine (AgSu) may be used topically for preventing infections in severe burns (Russell, 1985).

As might be imagined, silver nitrate is a somewhat astringent compound, below 10^{-4} mol/l a protein precipitant, and attempts to reduce this undesirable propensity while maintaining antimicrobial potency have been made. A device much used in pharmaceutical formulation to promote slow release of a potent substance is to combine it with a high-molecular-weight polymer. By mixing silver oxide or silver nitrate with gelatin or

albumen, a water-soluble adduct is obtained, which slowly releases silver ions but lacks the caustic astringency of silver nitrate. A similar slow-release compound has been prepared by combining silver with disodiumdinaphthylmethane disulphate (Goldberg *et al.*, 1950).

The oligodynamic action of silver (Langwell, 1932), already referred to in the historical introduction (Chapter 1) and above, has been exploited in a water purification system employing what is called katadyn silver. Here, metallic silver is coated on to sand used in filters for water purification. Silver-coated charcoal has been used in a similar fashion (Bigger & Griffiths, 1933; Gribbard, 1933; Brandes, 1934; Moiseev, 1934). The activity of a silver-releasing surgical dressing has been described by Furr *et al.* (1944), who used a neutralization system to demonstrate that Ag^+ ions releases were responsible for its antibacterial effects.

Russell & Hugo (1994) have reviewed the antimicrobial activity and action of silver compounds. At a concentration of 10^{-9} to 10^{-6} mol/l, Ag^+ is an extremely active biocide. Originally considered to act as a 'general protoplasmic poison', it is now increasingly seen that this description is an oversimplification. It reacts strongly with structural and functional thiol groups in microbial cells, induces cytological changes and interacts with the bases in DNA.

Silver sulphadiazine is essentially a combination of two antibacterial agents, Ag^+ and sulphadiazine. It has a broad spectrum of activity, produces surface and membrane blebs and binds to various cell components, especially DNA (reviewed by Russell & Hugo, 1994), although its precise mode of action has yet to be elucidated. Silver sulphadiozine has been reinvestigated by Hamilton-Miller *et al.* (1993).

15.3 Mercury compounds

Mercury, long a fascination for early technologists (alchemists, medical practitioners, etc.), was used in medicine by the Arabian physicians. In the 1850s, mercury salts comprised, with phenol, the hypochlorites and iodine, the complement of topical antimicrobial drugs at the physician's disposal. Mercuric chloride was used and evaluated by Robert Koch and by Geppert. Nowadays its use in medicine has decreased, although a number of organic derivatives of mercury (Fig. 2.27) are used as bacteriostatic and fungistatic agents and as preservatives and bactericides in injections; examples include mercurochrome, nitromersol, thiomersal and phenylmercuric nitrate (Fig. 2.27). Salts such as the stearate, oleate and naphthenate were, until much more recently, extensively employed in the preservation of wood, textiles, paints and leather, to quote a few examples (Table 2.15). With the advent of a major health disaster in Japan due to mercury waste, feeling is hardening all over the world against the use of mercury in any form where it might pollute the environment, and it is unlikely that the inclusion of mercury in any product where environmental pollution may ensue will be countenanced by regulatory authorities.

Mercury resistance is inducible and is not the result of training or tolerance. Plasmids conferring resistance are of two types: (i) 'narrow-spectrum', encoding resistance to Hg(II) and to a few specified

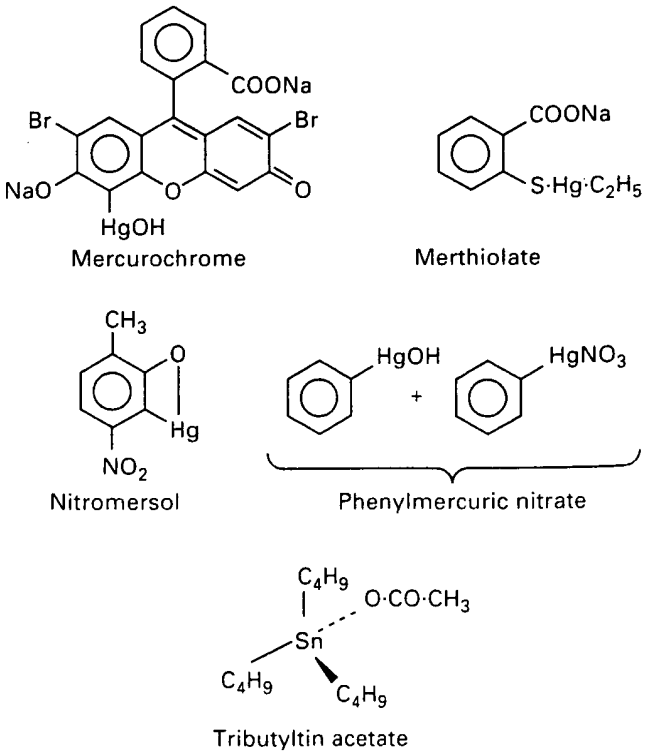


Fig. 2.27 Mercurochrome, merthiolate (thiomersal, sodium ethylmercurithiosalicylate), nitromersol, phenylmercuric nitrate and tributyltin acetate.

Table 2.15 Derivatives of mercury and their uses as preservatives.

Compound	Use(s)
Phenylmercuric stearate	Leather
Phenylmercuric oleate	Leather
Mercuric naphthenate	Paint
Phenylmercuric acetate	Papers, textiles, pharmaceuticals*
Phenylmercuric nitrate	Pharmaceuticals*

*For additional information, see Chapter 16.

organomercurials; and (ii) 'broad-spectrum', encoding resistance to those in (i) plus other organomercury compounds (Foster, 1983). In (i) there is enzymatic reduction of mercury to Hg metal and its vaporization, and in (ii) there is enzymatic hydrolysis of an organomercurial to inorganic mercury and its subsequent reduction as in (i) (Silver & Misra, 1988). Further details are provided in Chapter 10B and by Russell & Chopra (1996).

Mercury is an environmental pollutant of considerable concern because it is very toxic to living cells. Ono *et al.* (1988) showed that the yeast cell wall acted as an adsorption filter for Hg^+. Later (Ono *et al.*, 1991) they demonstrated that methylmercury-resistant mutants of *S. cerevisiae* overproduced hydrogen sulphide, with an accumulation of hydrosulphide (HS^-) ions intracellularly, which was responsible for detoxification of methylmercury.

15.3.1 Mercurochrome (disodium-2,7-dibromo-4-hydroxymercurifluorescein)

This is now only of historical interest; it was the first organic mercurial to be used in medicine and an aqueous solution enjoyed a vogue as a substitute for iodine solutions as a skin disinfectant.

15.3.2 Nitromersol (anhydro-2-hydroxymercuri-6-methyl-3-nitrophenol)

A yellow powder, it is not very soluble in water or organic solvents but will dissolve in aqueous alkali, and is used as a solution of the sodium salt. It is active against vegetative microorganisms but ineffective against spores and acid-fast bacteria. It is mostly used in the USA.

15.3.3 Thiomersal (merthiolate; sodium-o-(ethylmercurithio)-benzoate)

This derivative was used as a skin disinfectant, and is now employed as a fungicide and as a preservative (0.01–0.02%) for biological products, for example, bacterial and viral vaccines. It possesses antifungal properties but is without action on spores.

Solutions are stable when autoclaved but less stable when exposed to light or to alkaline conditions, and they are incompatible with various chemicals, including heavy-metal salts (Denyer & Wallhäusser, 1990).

15.3.4 Phenylmercuric nitrate (PMN)

This organic derivative is used as a preservative in multidose containers of parenteral injections and eye-drops at a concentration of 0.001% and 0.002% w/v, respectively (Brown & Anderson, 1968). It was formerly employed in the UK as an adjunct to heat in the now-discarded process of 'heating with a bactericide'.

Phenylmercuric nitrate is incompatible with various compounds, including metals. Its activity is reduced by anionic emulsifying and suspending agents (Denyer & Wallhäusses, 1990). Sulphydryl agents are used as neutralizers in bactericidal studies and in sterility testing (Russell *et al.*, 1979; Sutton, 1996). Phenylmercuric nitrate is a useful preservative and is also employed as a spermicide.

Phenylmercuric nitrate solutions at room temperature are ineffective against bacterial spores, but they possess antifungal activity and are used as antifungal agents in the preservation of paper, textiles and leather. Voge (1947) has discussed PMN in a short review. An interesting formulation

Table 2.16 Tin compounds used as preservatives and some examples of their uses.

Compound	Chemical formula	Use(s)
Tributyltin oxide	$((C_4H_9)_3\ Sn)_2O$	Antifouling paints Wallpaper adhesives Wood preservatives Antislime agents
Tributyltin fluoride	$(C_4H_9)_3\ SnF$	Antifouling paints
Tributyltin acetate	$(C_4H_9)_3\ SnOCOCH_3$	Antifouling paints
Tributyltin benzoate	$(C_4H_9)_3\ SnOCOC_6H_5$	Germicide: usually used with formaldehyde or a QAC
Triphenyltin acetate	$(C_6H_5)_3SnOCOCH_3$	Agricultural fungicides
Triphenyltin hydroxide	$(C_6H_5)_3SnOH$	Agricultural pesticides Disinfectants

of PMN with sodium dinaphthylmethanedisulphonate has been described, in which enhanced activity and greater skin penetration is claimed (Goldberg *et al.*, 1950).

15.3.5 Phenylmercuric acetate (PMA)

This has the same activity, properties and general uses as PMN (Denyer & Wallhäusser, 1990) and finds application as a preservative in pharmaceutical and other fields.

15.4 Tin and its compounds (organotins)

Tin, stannic or tin(IV) oxide was at one time used as an oral medicament in the treatment of superficial staphylococcal infections. Tin was claimed to be excreted via sebaceous glands and thus concentrated at sites of infection. More recently, organic tin derivatives (Table 2.16, Fig. 2.27) have been used as fungicides and bactericides and as textile and wood preservatives (Smith & Smith, 1975).

The organotin compounds which find use as biocides are derivatives of tin(IV). They have the general structure R_3SnX where R is butyl or phenyl and X is acetate, benzoate, fluoride, oxide or hydroxide. In structure–activity studies, activity has been shown to reside in the R group; the nature of X determines physical properties such as solubility and volatility (Van der Kerk & Luijten, 1954; Rose & Lock, 1970). The R_3SnX compounds, with R = butyl or phenyl, combine high biocidal activity with low mammalian toxicity. Samples of the range of R_3SnX compounds and their use as biocides are shown in Tables 2.16 and 2.17. Tin differs significantly from copper, silver and mercury salts in being intrinsically much less toxic. It is used to coat cans and vessels used to prepare food or boil water. Organotin compounds have some effect on oxidative phosphorylation (Aldridge & Threlfall, 1961) and act as ionophores for anions (Chapter 9). Possible environmental toxicity should be borne in mind when tin compounds are used.

Table 2.17 Minimum inhibitory concentrations (MICs) of tributyltin oxide towards a range of microorganisms.

Organism	MIC (μg/ml)
Aspergillus niger	0.5
Chaetomium globosum	1.0
Penicillium expansum	1.0
Aureobasidium pullulans	0.5
Trichoderma viride	1.0
Candida albicans	1.0
Bacillus mycoides	0.1
Staphylococcus aureus	1.0
Bacterium ammoniagenes	1.0
Pseudomonas aeruginosa	> 500
Enterobacter aerogenes	> 500

16 Anilides

Anilides (Fig. 2.28) have the general structure $C_6H_5.NH.COR$. Two derivatives—salicylanilide, where $R = C_6H_4OH$, and diphenylurea (carbanilide), where $R = C_6H_5.NH$—have formed the basis for antimicrobial compounds.

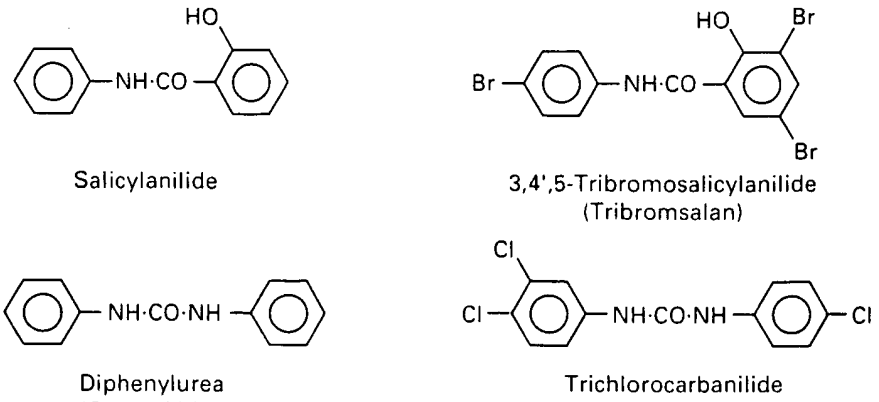


Fig. 2.28 Anilides.

16.1 Salicylanilide

The parent compound, salicylanilide, was introduced in 1930 as a fungistat for use on textiles (Fargher *et al.*, 1930). It occurs as white or slightly pink crystals, m.p. 137°C, which are soluble in water and organic solvents. It has also been used in ointment form for the treatment of ringworm, but concentrations above 5% should not be used in medicinal products because of skin irritancy. Minimum inhibitory concentrations (μg/ml) for a number of fungi were: *Trichophyton mentagrophytes*, 12; *Trichophyton tonsurans*, 6; *Trichophyton rubrum*, 3; *Epidermophyton floccosum*, 6; *Microsporum adovini*, 1.5. Despite the effectiveness of the parent compound, attempts were made to improve on its performance by the usual device of adding substituents, notably halogens, to the benzene residues; these are considered below.

16.1.1 Substituted salicylanilides

Lemaire *et al.* (1961) investigated 92 derivatives of salicylanilide and related compounds, i.e. benzanilides and salicylaldehydes. The intrinsic antimicrobial activity was obtained from literature values and was usefully summarized as follows. One ring substituent would give an MIC value for *S. aureus* of 2 μg/ml, but this value could be decreased to 1 μg/ml if substitution occurred in both rings.

The researchers were particularly interested in the role of these compounds as antiseptics for addition to soaps, and went on to evaluate them in this role. They were also interested to find to what extent they remained on the skin (skin substantivity) after washing with soaps containing them. They found that di- to pentachlorination or bromination with more or less equal distribution of the substituent halogen in both rings gave the best results both for antimicrobial activity and skin substantivity. However, it was also found that skin photosensitization was caused by some analogues.

Of the many compounds tested, the 3,4′,5-tribromo, 2,3,5,3′- and 3,5,3′,4′-tetrachloro salicylanilides have been the most widely used as antimicrobial agents; however, their photosensitizing properties have tended to restrict their use in any situation where they may come in contact with human skin.

Over and above this, many workers who have investigated germicidal soaps, i.e. ordinary soap products with the addition of a halogenated salicylanilide, carbanilide, or for that matter phenolic compounds such as hexachlorophane (2.9.1) or DCMX (2.6.5), have doubted their value in this role, although some may act as deodorants by destroying skin organisms which react with sweat to produce body odour.

16.2 Diphenylureas (carbanilides)

16.2.1 3,4,4′-Trichlorocarbanilide (TCC, triclocarban)

From an extensive study by Beaver *et al.* (1957), the above emerged as one of the most potent of this family of biocides. It inhibits the growth of many Gram-positive bacteria at concentrations from 0.1 to 1.0 μg/ml. Fungi were found to be more resistant, since 1000 μg/ml failed to inhibit *A. niger, Penicillium notatum, C. albicans* and

Fusarium oxysporium. *Trichophyton gypseum* and *Trichophyton inguinale* were inhibited at 50 µg/ml.

It occurs as a white powder, m.p. 250°C; it is very slightly soluble in water.

Like the salicylanilides, it has not found favour in products likely to come in contact with human skin, despite the fact that it had been extensively evaluated as the active ingredient of some disinfectant soaps.

16.3 Mode of action

The mode of action of salicylanilides and carbanilides (diphenylureas) has been studied in detail by Woodroffe & Wilkinson (1966a,b) and Hamilton (1968). The compounds almost certainly owe their bacteriostatic action to their ability to discharge part of the proton-motive force, thereby inhibiting processes dependent upon it, i.e. active transport and energy metabolism. Further general details will be found by consulting Chapter 9 and Russell & Chopra (1996).

17 Miscellaneous preservatives

Included in this section are those chemicals which are useful preservatives but which do not form part of the biocidal groups already discussed.

17.1 Derivatives of 1,3-dioxane

17.1.1 6-Acetoxy-2,4-dimethyl-1,3-dioxane (dimethoxane) (Dioxin: registered trade mark, Sindar Corporation, New York, USA)

Dioxin (Fig. 2.29) is a liquid, colourless when pure and soluble in water and organic solvents. It has a marked odour. It is active against a wide range of microorganisms at concentrations ranging from 300 to 2500 µg/ml (Anon., 1962). It should be noted that the name 'dioxin' is also used for a reaction product, 2,3,7-8-tetrachlorodibenzo-*p*-dioxin (TCDD), which may be formed during the manufacture of trichlorophenol. The MIC values (µg/ml) for representative microorganisms are: *S. cerevisiae*, 2500; *A. niger*, 1250; *S. aureus*, 1250; *P. aeruginosa*, 625; *Salmonella cholerae-suis*, 312.

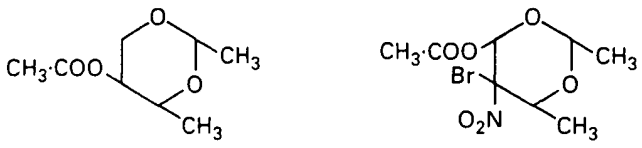


Fig. 2.29 Dioxanes: dioxin and bronidox.

Dimethoxane is not affected by changes in pH but it is slowly hydrolysed in aqueous solution, producing ethanal (acetaldehyde). It is compatible with non-ionic surface-active agents but may cause discoloration in formulations that contain amines or amides.

Dimethoxane finds application as a preservative for cosmetics, emulsion paints and cutting oils. A detailed study of the components of the commercial preparation Giv Gard DXN (Givaudan & Co. Ltd., Whyteleafe, Surrey, UK) showed that the acetoxy group may be either 6-α (74%) or 6-β (22%) to the 1,3-dioxane ring. Small amounts of acetaldehyde may also be present (Woolfson & Woodside, 1976). Later, in a bacteriological study, Woolfson (1977) attributed the action of the commercial product partially to its aldehyde content and partially to the 1,3-dioxane components.

17.1.2 5-Bromo-5-nitro-1,3-dioxane (Bronidox: Henkel Chemicals Ltd, Tretol House, London NW9 0HT, UK)

This nitro–bromo derivative of dioxane is available as a 10% solution in propylene glycol as Bronidox L. It is used as a preservative for toiletries and has been described in some detail by Potokar *et al.* (1976) and Lorenz (1977). Its stability at various pH values is tabulated by Croshaw (1977).

It is active against bacteria and fungi and does not show a *Pseudomonas* gap. Minimum inhibitory concentrations of the active ingredient (µg/ml) were: *E. coli*, 50; *P. aeruginosa*, 50; *P. vulgaris*, 50; *P. fluorescens*, 50; *S. typhi*, 50; *Serratia marcescens*, 25; *S. aureus*, 75; *S. faecalis*, 75; *C. albicans*, 25; *S. cerevisiae*, 10; *A. niger*, 10.

Its activity is not affected between pH 5 and 9 and it probably acts as an oxidizing agent, oxidizing –SH to –S–S– groups in essential enzymes. It does not act as a formaldehyde releaser.

It is recommended for use as a preservative for a variety of toiletries, including shampoos and hand lotions.

17.2 Derivatives of imidazole

Imidazolines (Fig. 2.30) are 2,3-dihydroimidazoles; 2-heptadecyl-2-imidazoline was introduced as an agricultural fungicide as far back as 1946. Other derivatives containing the imidazole ring have recently found successful application as preservatives. Two are derivatives of 2,4-dioxotetrahydroimidazole, the imidazolidones; the parent diketone is hydantoin.

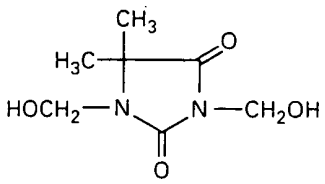
17.2.1 1,3-Di(hydroxymethyl)-5,5-dimethyl-2,4-dioxoimidazole; 1,3-Di-hydroxymethyl)-5,5-dimethylhydantoin (Dantoin)

A 55% solution of this compound (Fig. 2.30) is available commercially as Glydant (Glyco Chemicals Inc., Greenwich, Conn., USA). This product is water-soluble, stable and non-corrosive, with a slight odour of formaldehyde. It is active over a wide range of pH and is compatible with most ingredients used in cosmetics. It has a wide spectrum of activity against bacteria and fungi, being active at concentrations of between 250 and 500 µg/ml. The moulds *Microsporum gypseum* and *Trichophyton asteroides*, however, are particularly susceptible, being inhibited at 32 µg/ml. Its mode of action is attributed to its ability to release formaldehyde, the rate of release of which is more rapid at high pH values, 9–10.5, than low, 3–5. Its optimum stability lies in the range pH 6–8. It has an acceptable level of toxicity and can be used as a preservative over a wide field of products. It has been evaluated by Schanno *et al.* (1980).

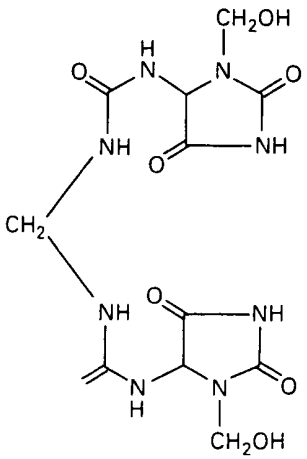
17.2.2 N,N''-methylene bis [5'[1-hydroxymethyl] -2,5-dioxo-4-imidazolidinyl urea] (Germall 115: Sutton Laboratories Inc., Roselle, N.J., USA)

In 1970 a family of imidazolidinyl ureas for use as preservatives was described (Berke & Rosen, 1970). One of these, under the name Germall 115, has been studied extensively (Rosen & Berke, 1973; Berke & Rosen, 1978). Germall 115 is a white powder very soluble in water, and hence tends to remain in the aqueous phase of emulsions. It is non-toxic, non-irritating and non-sensitizing. It is compatible with emulsion ingredients and with proteins.

A claimed property of Germall 115 has been its ability to act synergistically with other preservatives (Jacobs *et al.*, 1975; Rosen *et al.*, 1977; Berke & Rosen, 1980). Intrinsically it is more active against bacteria than fungi. Most of the microbiological data are based on challenge tests in cosmetic formulations, data which are of great value to the cosmetic microbiologist. An investigation of its activity against a series of *Pseudomonas* species and strains (Berke & Rosen, 1978) showed that in a challenge test 0.3% of the compound cleared all species but *P. putida* and *P. aureofaciens* in 24 h. The latter species were killed between 3 and 7 days. In an agar cup-plate test, 1% solution gave the following size inhibition zones (mm): *S. aureus*, 7,6; *S. aureus*, penicillin sensitive, 15.5; *Staphylococcus albus*, 9.0; *B. subtilis*, 15.0; *Corynebacterium. acne*, 5.0; *E. coli*, 3.6; *Pseudomonas ovale*, 2.0.



Glydant DM DMH–55



Germall 115

Fig. 2.30 Dantoin or Glydant DMDMH-55 and Germall 115.

17.3 Isothiazolones

Ponci *et al.* (1964) studied the antifungal activity of a series of 5-nitro-1,2-dibenzisothiazolones and found many of them to possess high activity. Since this publication a number of isothiazolones (Fig. 2.31) have emerged as antimicrobial preservatives. They are available commercially, usually a suspensions rather than as pure compounds, and find use in a variety of industrial situations. Nicoletti *et al.* (1993) have described their activity.

17.3.1 5-Chloro-2-methyl-4-isothiazolin-3-one (CMIT)

17.3.2 2-Methyl-4-isothiazolin-3-one (MIT)

A mixture of these two derivatives, known as Kathon 886 MW (Rohm and Haas (UK) Ltd., Croydon, CR9 3NB, UK), containing about 14% of active ingredients is available as a preservative for cutting oils and as an in-can preservative for emulsion paints. This mixture is active at concentrations of 2.25–9 μg/ml active ingredient against a wide range of bacteria and fungi and does not show a *Pseudomonas* gap. It is also a potent algastat. Kathon CG, containing 1.5% active ingredients and magnesium salts, has been suggested as a preservative for cosmetic products up to a final concentration of 25 μg/ml active ingredients.

It possesses the additional advantage of being biodegradable to non-toxic metabolites and is non-irritating at normal in-use concentrations. It is water-soluble and compatible with most emulgents. The stability of Kathon 886 at various pH values is described by Croshaw (1977).

17.3.3 2-n-Octyl-4-isothiazolin-3-one (Skane M8: ICI)

This is available as a 45% solution in propylene glycol and is active against bacteria over a range of 400–500 μg/ml active ingredient. To inhibit the growth of one strain of *P. aeruginosa* required 500 μg/ml. Fungistatic activity was shown against a wide number of species over the range 0.3–8.0 μg/ml. It is also effective at preventing algal growth at concentrations of 0.5–5.0 μg/ml. It is biodegradable but shows skin and eye irritancy. As might be expected from its *n*-octyl side-chain, it is not soluble in water.

17.3.4 1,2-Benzisothiazolin-3-one (BIT). (Proxel CRL, GXL, AB: Imperial Chemical Industries Ltd., Blackley, Manchester M9 3DA)

This is available commercially in various formulations and is recommended as a preservative for industrial emulsions, adhesives, polishes, glues and paper products. It possesses a low mammalian toxicity but is not recommended for medicinal and cosmetic use for it exhibits marked skin irritancy.

17.3.5 Mechanism of action

As growth-inhibitory concentrations, BIT has little effect on the membrane integrity of *Staph. aureus*, but significantly inhibits active transport and oxidation of glucose and has a marked effect on thiol-containing enzymes.

Thiol-containing compounds quench the activity of BIT, CMIT and MIT against *E. coli*, which suggests that these isothiazolones interact strongly with –SH groups. The activity of CMIT is also overcome by non-thiol amino acids, so that this compound might thus react with amines as well as with essential thiol groups (Collier *et al.*, 1990a,b).

17.4 Derivatives of hexamine

Hexamine (hexamethylene tetramine; 1,3,5,7-triaza-1-azonia-adamantane) has been used as a

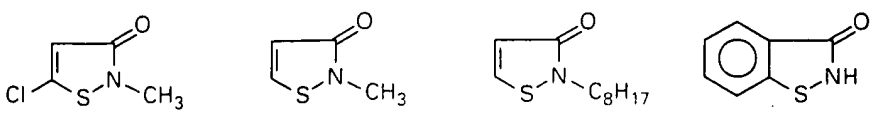


Fig. 2.31 Isothiazolones. From left to right: 5-chloro-2-methyl-4-isothiazolin-3-one (CMIT, Section 17.3.1 in text), 2-methyl-4-isothiazolin-3-one (MIT; 17.3.2), 2-*n*-octyl-4-isothiazolin-3-one (17.3.3) and 1,2-benzisothiazolin-3-one (BIT; 17.3.4).

Table 2.18 Inhibitory concentrations for hexamine quaternized with $-CH_2Cl=CHCl$ compared with values for hexamine and formaldehyde.

Inhibitor	MIC* against					
	Staph. aureus	*Sal. typhi*	*K. aerogenes*	*Ps. aeruginosa*	*B. subtilis*	*D. desulphuricans*
Hexamine quaternized with $-CH_2-CH=CHCl$	4×10^{-4} (100)	2×10^{-4} (50)	2×10^{-4} (50)	2×10^{-3} (500)	4×10^{-4} (100)	2.9×10^{-2} (7250)
Hexamine	3.5×10^{-2} (5000)	3.5×10^{-3} (500)	–	–	–	5.3×10^{-2} (7500)
Formaldehyde	1.6×10^{-3} (50)	3.3×10^{-3} (100)	1.6×10^{-3} (50)	–	–	–

*Molar values (in parentheses μg/ml).

Table 2.19 Properties of the most commonly used gaseous disinfectants.

Gaseous disinfectant	Molecular weight	Boiling point (°C)	Solubility in water	Sterilizing concn (mg/l)	Relative humidity requirements (%)	Penetration of materials	Microbidical activity*	Best application as gaseous disinfectant†
Ethylene oxide	44	10.4	Complete	400–1000	Non-desiccated 30–50; large load 60	Moderate	Moderate	Sterilization of plastic medical supplies
Propylene oxide	58	34	Good	800–2000	Non-desiccated 30–60	Fair	Fair	Decontamination
Formaldehyde	30	90°C/ Formalin‡	Good	3.10	75	Poor (surface sterilant)	Excellent	Surface sterilant for rooms
β-Propiolactone	72	162	Moderate	2–5	>70	None (surface sterilant)	Excellent	Surface sterilant for rooms
Methyl bromide	95	4.6	Slight	3500	30–50	Excellent	Poor	Decontamination

*Based on an equimolar comparison.
†See later also, Chapter 21.
‡Formalin contains formaldehyde plus methanol.

urinary antiseptic since 1894. Its activity is attributed to a slow release of formaldehyde. Other formaldehyde-releasing compounds are considered in Sections 7.2.4, 17.2, 17.5 and 17.6. Wohl in 1886 was the first to quaternize hexamine, and in 1915–16 Jacobs and co-workers attempted to extend the antimicrobial range of hexamine by quaternizing one of its nitrogen atoms with halo-hydrocarbons (Jacobs & Heidelberger, 1915a,b; Jacobs *et al.*, 1916a,b). These workers did not consider that their compounds acted as formaldehyde releasers but that activity resided in the whole molecule.

The topic was taken up again by Scott & Wolf (1962). These workers re-examined quaternized hexamine derivatives with a view to using them as preservatives for toiletries, cutting oils and other products. They looked at 31 such compounds and compared their activity also with hexamine and formaldehyde. As well as determining their inhibitory activity towards a staphylococcus, enterobacteria and a pseudomonad, they also assessed inhibitory activity towards *Desulphovibrio desulphuricans*, a common contaminant of cutting oils.

Polarographic and spectroscopic studies of formaldehyde release were made on some of the derivatives; this release varied with the substituent used in forming the quaternary salt. A typical set of data for the antimicrobial activity (MIC) of one derivative compared with hexamine and formaldehyde is shown in Table 2.18. In general, the quaternized compounds were found to be more active w/w than hexamine but less active than formaldehyde. Although chemically they contain a quaternized nitrogen atom, unlike the more familiar antimicrobial quaternized compounds (Section 6.1), they are not inactivated by lecithin or protein. The compounds are not as surface-active as conventional QACs. Thus an average figure for the surface tension, dyne cm^{-1}, for 0.1% solutions of the quaternized hexamines was 54; that for 0.1% cetrimide (Section 6.1) was 34.

One of these derivatives of hexamine, i.e. that quaternized with *cis*-1,3-dichloropropene, is being used as a preservative under the name Dowicil 200 (Dow Chemical Co., Wilmslow, Cheshire, UK). *Cis*-1-(3-cis-chloroallyl)-3,5,7-triaza-1-azonia-admantane chloride *N*-(3-chloroallyl) hexamine (Dowicil 200; Fig. 2.32) is a highly water-soluble

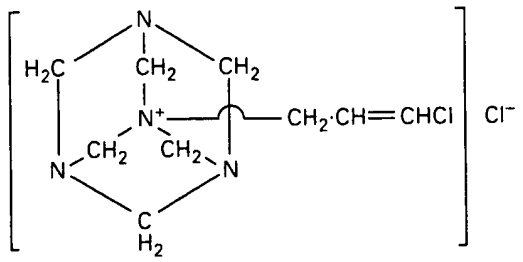


Fig. 2.32 Dowicil 200 (*N*-(3-*cis*-chloroallyl)hexamine).

hygroscopic white powder; it has a low oil solubility. It is active against bacteria and fungi. Typical MIC (μg/ml) were: *E. coli*, 400; *P. vulgaris*, 100; *S. typhi*, 50; *Alcaligenes faecalis*, 50; *P. aeruginosa*, 600; *S. aureus*, 200; *B. subtilis*, 200; *A. niger*, 1200; *T. interdigitale*, 50.

It is recommended for use as a preservative for cosmetic preparations at concentrations of from 0.1 to 0.2%. Because of its high solubility, it does not tend to concentrate in the oil phase of these products, but remains in the aqueous phase, where contamination is likely to arise. It is not inactivated by the usual ingredients used in cosmetic manufacture. Its activity is not affected over the usual pH ranges found in cosmetic or cutting oil formulations. For further information, see Rossmore & Sondossi (1988).

17.5 Triazines

The product, theoretically from the condensation of three molecules of ethylamine with three of formaldehyde, is hexahydro-1,3,5-triethyl-*s*-triazine (Bactocide THT: Cochrane and Keene (Chemicals), Rochdale, UK; Fig. 2.33a). This is a clear white or slightly yellow viscous liquid, readily soluble in water, acetone, ethanol and ether. It is bactericidal and fungicidal and inhibits most bacteria, including *P. aeruginosa* and *D.*

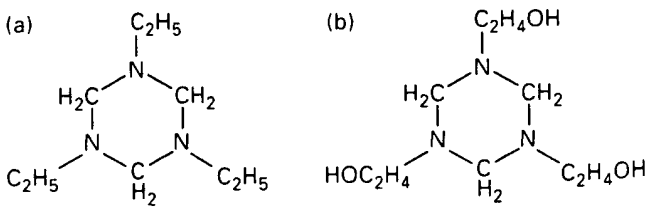


Fig. 2.33 (a) Hexahydro-1,3,5-triethyl-*s*-triazine (Bactocide THT): (b) 1,3,5-tris(2-hydroxyethyl)-*s*-triazine (Grotan).

desulphuricans at concentrations of 0.3 mg/ml. Fungi, such as *A. niger*, *Penicillium glaucum* and *P. notatum* are inhibited at 0.1 mg/ml, and *Sacch. cerevisiae* at 0.05 mg/ml. It owes its activity to a release of formaldehyde. It has been used as a preservative for cutting oils, for the 'in-can' preservation of emulsion paints for proteinaceous adhesives and to control slime in paper and cardboard manufacture, and to prevent the growth of microorganisms in water-cooling systems. It has a low intrinsic toxicity and at use dilutions is not irritant to the skin.

If formaldehyde is reacted with ethanolamine, the compound 1,3,5-tris(2-hydroxyethyl)-*s*-triazine can be formed (Grotan: Stirling Industrial, Sheffield, UK; Fig. 2.33b). This has both antibacterial and antifungal activity and is recommended as a preservative for cutting oils. Despite the figures for fungal inhibition, it is often found, in practical preservation situations, that, although this triazine will inhibit microbial growth, a fungal superinfection is often established; a total preservation system which includes a triazine might well have to contain an additional antifungal compound (Rossmore *et al.*, 1972; Paulus, 1976). This situation may be compared with that found with imidazole derivatives (Section 17.2).

Rossmore (1979) has discussed the uses of heterocyclic compounds as industrial biocides, and Rossmore & Sondossi (1988) have reviewed formaldehyde condensates in general.

17.6 Oxazolo-oxazoles

By reacting formaldehyde with tris(hydroxymethyl)-methylamine, a series of derivatives is obtained. The commercial product (Nuosept 95: Tenneco Organics Ltd., Avonmouth, Bristol, UK; Fig. 2.34) contains the molecules species: 5-hydroxymethoxymethyl-1-aza-3,7-dioxabicyclo (3.3.0) octane, 24.5%; 5-hydroxymethyl-1-aza-3,7-dioxabicyclo (3.3.0) octane, 17.7%; 5-hydroxypolymethylenoxy (74% C_2, 21% C_3, 4% C_4, 1% C_5) methyl-1-aza-3,7-dioxabicyclo (3.3.0) octane, 7.8%, and acts as a biostat by virtue of being a formaldehyde releaser.

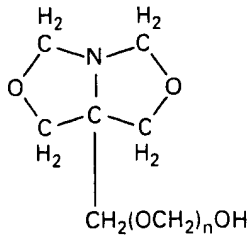


Fig. 2.34 Nuosept 95 (n = 0–5).

It is obtained as a clear, pale-yellow liquid, which is miscible with water, methanol, ethanol, chloroform and acetone in all proportions, and is recommended as a preservative for cutting oils, water treatment, plants, emulsion (latex) paints, industrial slurries and starch- and cellulose-based products. It is slightly irritant to intact and abraded skin and is a severe eye irritant.

17.7 Methylene bisthiocyanate

This is available commercially as a 10% solution and is recommended for the control of slime in paper manufacture, where it provides a useful alternative to mercurials. The compound (Fig. 2.35) is a skin and eye irritant and thus care is required in its use. Its toxicity is low enough to enable it to be used in the manufacture of papers destined for the packaging of food. At in-use dilutions, it is unlikely to cause corrosion of materials used in the construction of paper-manufacturing equipment.

17.8 Captan

Captan is *N*-(trichloromethylthio)cyclohex-4-ene-1,2-dicarboximide (Fig. 2.36). It is a white crystalline solid, insoluble in water and only slightly

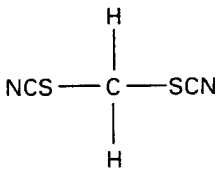


Fig. 2.35 Methylene bisthiocyanate.

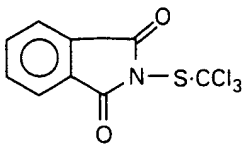


Fig. 2.36 Captan.

soluble in organic solvents. It is decomposed in alkaline solution. Despite its low solubility, it can be shown to be an active biocide, being active against both Gram-negative and Gram-positive bacteria, yeasts and moulds. It has been used as an agricultural fungicide, being primarily employed against diseases of fruit trees. It has also been used to prevent spoilage of stored fruit and in the treatment of skin infections due to fungi in humans and animals.

17.9 Essential oils

Essential oils have been used empirically throughout history as preservatives. Their re-examination as antimicrobial agents has received attention from many workers, as their use as natural preservatives has contemporary appeal. Their antibacterial properties have been reviewed by Deans & Ritchie (1987).

17.10 General statement

Many of these compounds are relatively new in the preservation field and much of the information concerning their properties and uses is found in the manufacturers' information brochures. Any person wishing to explore their use should consult the manufacturers. An ever-present problem with cosmetics preservation is that of contact sensitization. This is discussed in some detail by Marzulli & Maibach (1973) and is a point which must be carefully checked before a preservative is committed to a product. Another hazard which may arise is that of an induced change in the skin microflora during continuous use of products containing antimicrobial preservatives; this is discussed by Marples (1971).

18 Vapour-phase disinfectants

Gaseous sterilization is the subject of a later chapter (Chapter 21) and thus only a few comments will be made here. It is only comparatively recently that a scientific basis for using gases as sterilizing or disinfecting agents has been established. Factors influencing the activity of gaseous formaldehyde were described by Nordgren (1939) and later by a Committee on Formaldehyde Disinfection (Anon., 1958). The possible uses of gaseous formaldehyde in the disinfection of hospital bedding and blankets and, in conjunction with low-temperature steam, for disinfection of heat-sensitive material, are considered in Section 18.2.1 (see also Chapter 21).

Phillips & Kaye (1949) reviewed the earlier work which had taken place with ethylene oxide, which has bactericidal, mycobactericidal, sporicidal, fungicidal and viricidal activity (Ernst, 1974). A later review is by Richards *et al.* (1984).

Other gases of possible value include β-propiolactone, propylene oxide, ozone, methyl bromide and glycidaldehyde (Russell, 1976). Physical and chemical properties of these and the two most important ones (ethylene oxide and formaldehyde) are listed in Table 2.19 and their chemical structures are given in Fig. 2.37.

Gaseous hydrogen peroxide and gas plasmas are likely to play an important role as sterilizing agents in the future.

18.1 Ethylene oxide

This is discussed in detail later (Chapter 21) and will not be considered here in detail. Its antibicrobial activity is affected by concentration, temperature, relative humidity and the water content of microorganisms. It acts, by virtue of its alkylating properties, on proteins and nucleic acids. A consideration of its antimicrobial activity with compounds of a similar chemical structure (Figs 2.37 and 2.38) demonstrates that cyclopropane, which is not an alkylating agent, is not antimicrobial whereas those that have the ability to alkylate are potent antimicrobials.

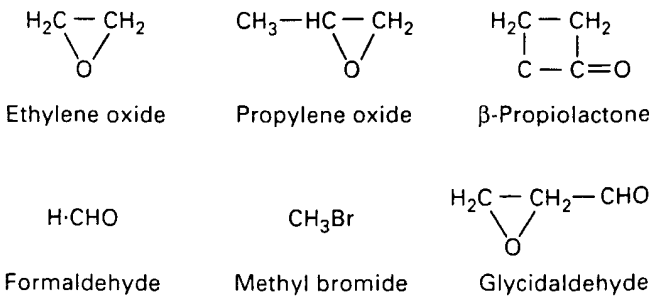


Fig. 2.37 Chemical structures of gaseous disinfectants.

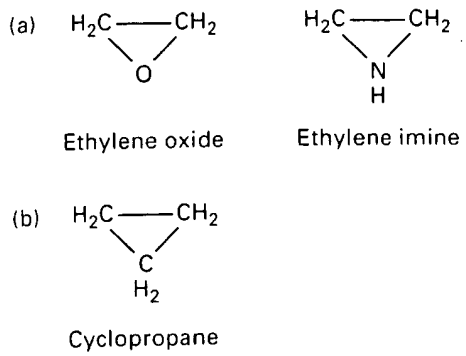


Fig. 2.38 Compounds similar to ethylene oxide: (a) alkylating and antimicrobial compounds; (b) non-alkylating, non-antimicrobial agent.

Useful reviews are those by Hoffman (1971), Phillips (1977), Richards *et al.* (1984), Burgess & Reich (1993), Jorkasky (1993), Page (1993) and Sintim-Damoa (1993).

18.2 Formaldehyde-releasing agents

Paraformaldehyde ($HO(CH_2O)_n.H$, where n = 8–100) is a polymer of formaldehyde and is produced by evaporating aqueous solutions of formaldehyde. Although it was considered originally to be of little practical use (Nordgren, 1939) paraformaldehyde has since been shown to depolymerize rapidly when heated, to produce formaldehyde (Taylor *et al.*, 1969). Paraformaldehyde is considered by Tulis (1973) to be an excellent source of monomeric formaldehyde gas, because it can be produced in a temperature-controlled reaction, and there are no contaminating residues (methanol and formic acid) produced during evaporation of formalin solutions, in contrast to the method of evaporating formalin solutions containing 10% methanol to prevent polymerization.

Other formaldehyde-releasing agents are melamine formaldehyde and urea formaldehyde (Fig. 2.39). The former is produced from formaldehyde and melamine under alkaline conditions and the latter is a mixture of monomethyloyl urea and dimethyloyl urea. When exposed to elevated temperatures these agents release potentially sterilizing amounts of gaseous formaldehyde, the rate of release being a function of time and temperature. These formaldehyde-releasing agents are, however, much less effective as disinfecting or sterilizing sources than paraformaldehyde. The

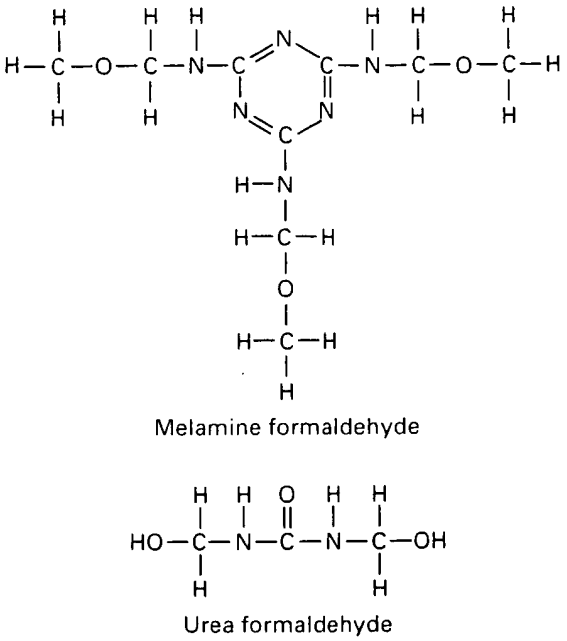


Fig. 2.39 Melamine formaldehyde and urea formaldehyde.

reason for this is that there is a much greater release of formaldehyde from paraformaldehyde than from the resins at various temperatures, and the microbicidal process is strictly a function of the available formaldehyde gas.

Applications and mode of action of formaldehyde-condensate biocides have been reviewed by Rossmore & Sondossi (1988) and Rossmore (1995).

18.2.1 Uses of formaldehyde vapour

Formaldehyde vapour has found use as a disinfectant in the following situations (Russell, 1976).

1 In combination with low-temperature steam (70–90°C) as a method for disinfecting heat-sensitive materials (Alder *et al.*, 1971, 1990). This will be discussed later (Chapter 19A); however, some recent studies (Wright *et al.*, 1996) have cast doubt on the efficacy of this process as a sterilization method because it has been possible by means of a post-heating shock to revive some treated spores.

2 Rarely, in the disinfection of hospital bedding and blankets, when formaldehyde solutions are used in the penultimate rinse of laundering blankets to give a residual bactericidal activity because of the slow evolution of formaldehyde

vapour (Dickinson & Wagg, 1967; Alder *et al.*, 1971, 1990).

3 In the terminal disinfection of premises, although this is considered to be of limited value (Kelsey, 1967).

4 As a fumigant in poultry houses after emptying and before new stock is introduced (Nicholls *et al.*, 1967; Anon., 1970) and in the hatcher to prevent bacterial contamination of shell eggs (Harry, 1963).

5 In the disinfection of safety cabinets.

18.3 Betapropiolactone

Betapropiolactone (BPL) requires heating to produce the vapour form, has weak penetrating powers (Table 2.19) and hydrolyses readily in water to give hydracrylic acid (β-hydroxy-propionic acid). Its antimicrobial activity depends primarily on its concentration and the temperature and r.h. at which it is used. Its antibacterial activity is maximal at r.h. levels of 75–80%, although, as with ethylene oxide, it is not so much the environmental moisture content that is important but the content and location of water within the bacterial cell (Hoffman & Warshowsky, 1958). The possibility of BPL being carcinogenic (Walpole *et al.*, 1954) has obviously limited its applications, although BPL vapour may have a use in the decontamination of premises (Spiner & Hoffman, 1960).

18.4 Propylene oxide

Propylene oxide requires only mild heating to produce the vapour form and has a fair penetration of materials (Table 2.19). It hydrolyses slowly in the presence of only a small amount of moisture to give the non-toxic propylene glycol (Kereluk, 1971) and there is no need to remove it from exposed materials (Sykes, 1965). Antibacterial activity decreases with an increase in r.h. (Bruch & Koesterer, 1961), although with desiccated organisms the reverse applies (Himmelfarb *et al.*, 1962). Propylene oxide has been shown to be suitable for treating powdered or flaked foods (Bruch & Koesterer, 1961).

18.5 Methyl bromide

Methyl bromide is a gas at normal temperatures. It is considerably less active as an antibacterial agent than ethylene oxide (Kelsey, 1967; Kereluk, 1971) or propylene oxide (Kelsey, 1967) but has good penetrative ability (Table 2.19). Methyl bromide is listed by Kereluk (1971) as being suitable for some types of fumigation.

18.6 Glycidaldehyde

Glycidaldehyde vapour inactives sporing and non-sporing bacteria; the inactivation rate depends on concentration, temperature and inversely on r.h. (Dawson, 1962). There is little information as to its possible usefulness as a disinfecting or sterilizing agent.

18.7 Ozone

Ozone, O_3, is an allotropic form of oxygen. It has powerful oxidizing properties, inhibits bacterial growth (Ingram & Haines, 1949; Baird-Parker & Holbrook, 1971) and is bactericidal, viricidal and sporicidal, although spores are 10–15 times more resistant than non-sporing bacteria (Gurley, 1985; Rickloff, 1985). Gaseous ozone reacts with amino acids, RNA and DNA. It is unstable chemically in water, but activity persists because of the production of free radicals, including $HO^{\bullet}$. A synergistic effect has been shown with the simultaneous use of sonication (Burleson *et al.*, 1975).

18.8 Carbon dioxide

Carbon dioxide in soft drinks inhibits the development of various types of bacteria (Dunn, 1968). The growth of psychrotolerant, slime-producing bacteria is markedly inhibited by CO_2 gas in the atmosphere (Clark & Lentz, 1969).

18.9 Mechanism of action

Only a few brief comments will be made, and the interested reader is directed to the reviews of Bruch & Bruch (1970), Hoffman (1971), Russell (1976) Richards *et al.* (1984) and Russell & Chopra (1996) for further information. As noted above

(Section 18.1, Figs. 2.37 and 2.38), there is strong evidence that ethylene oxide acts by virtue of its alkylating properties; this gaseous agent reacts with proteins and amino acids, and with nucleic acid guanine (to give 7-(2′-hydroxyethyl) guanine), with alkylation of phosphated guanine possibly being responsible for its activity (Michael & Stumbo, 1970). The N-7 guanine position may also be a primary reaction site for BPL (Troll *et al.*, 1969). Formaldehyde is an extremely reactive chemical, which interacts with cell protein, RNA and DNA (Russell & Hopwood, 1976).

19 Aerial disinfectants

An early procedure for aerial disinfection was the employment of sulphur dioxide, obtained by burning sulphur, or of chlorine for fumigating sickrooms.

An effective aerial disinfectant should be capable of being dispersed in the air so that complete and rapid mixing of infected air and disinfectant ensues. Additionally, an effective concentration should be maintained in the air, and the disinfectant must be highly and rapidly effective against airborne microorganisms at different relative humidities. To these microbiological properties must be added the property of no toxicity or irritancy.

The most important means of using aerial disinfectants is by aerosol production. Aerosols consist of a very fine dispersed liquid phase in a gaseous (air) disperse phase. The lethal action of aerosols is believed to be due to condensation of the disinfectant on to the microbial cell (Sykes, 1965). Thus, the disinfectant must be nebulized in a fine spray to enable it to remain airborne and thereby come into contact, by random collision, with any microorganisms present in the air. Aerosol droplets of $< 1\,\mu m$ tend to be the accepted standard. Relative humidity has an important bearing on activity and at low r.h. inadequate condensation of disinfectant on to the microbial cell occurs. This means that dust-borne organisms are less susceptible to aerial disinfectants than are those enclosed in droplets; the optimum r.h. is usually 40–60%. In practice, chemical aerosols may be generated by spraying liquid chemicals into the air from an atomizer; solids may be vaporized by heat from a thermostatically controlled hotplate or dissolved in an appropriate solid and atomized.

Various chemicals have been employed for disinfecting air, including the following.

1 Hexylresorcinol: this phenolic substance is active against a wide range of bacteria, but not spores, in air. It is vaporized from a thermostatically controlled hotplate, and the vapour is odourless and non-toxic.

2 Lactic acid: this is an effective bactericidal aerial agent, but is unfortunately irritant at high concentrations.

3 Propylene glycol: this may be employed as a solvent for dissolving a solid disinfectant prior to atomization, but is also a fairly effective and non-irritating antimicrobial agent in its own right (Baird-Parker & Holbrook, 1971).

4 Formaldehyde: in summary of previous information, formaldehyde gas may be generated by:

(a) evaporating commercial formaldehyde solution (formalin);

(b) adding formalin to potassium permanganate;

(c) volatilizing paraformaldehyde (Taylor *et al.*, 1969);

(d) exposing certain organic resins or polymers, such as melamine formaldehyde or urea formaldehyde, to elevated temperatures (Tulis, 1973; see Russell, 1976).

Fumigation by formaldehyde has found considerable use in poultry science (Anon., 1970).

20 Disinfectants in the food, dairy, pharmaceutical and cosmetic industries

The effectiveness of many disinfectants is reduced in the presence of organic matter in its various forms, such as blood, serum pus, dirt, earth, milkstone, food residues and faecal material (Chapter 3). This decreased activity has an important bearing on disinfectant use in the cosmetic (Davis, 1972a), pharmaceutical (Bean, 1967), food (Kornfeld, 1966; Goldenberg & Relf, 1967; Olivant & Shapton, 1970; Banner, 1995) and dairy (Clegg, 1967, 1970; Davis, 1972b; Anon., 1977) industries. The principles in all cases are the same, namely either adequate precleaning before use of the disinfectant or a combination of the disinfectant with a suitable detergent.

Organic matter may reduce activity either as a result of a chemical reaction between it and the compound, thus leaving a smaller antimicrobial concentration for attacking microorganisms, or through a protection of the organisms from attack (Sykes, 1965). Phospholipids in serum, milk and faeces will reduce the antimicrobial activity of QACs.

The nature of the surface being disinfected and the protection afforded by soiling film are of considerable importance, and invisible milkstone in the dairy industry may protect microorganisms against disinfection (Clegg, 1967). Rapid removal of soiling film may be achieved by use of high pH, for example the use of a combined hypochlorite-detergent at pH 11 (Clegg, 1967). Notwithstanding the lower activity of chlorine disinfectants at alkaline pH, an enhanced effect is observed because of the greater contact between microorganisms and disinfectant. Of course, under certain circumstances caustic soda solutions are themselves sporicidal (Clegg, 1970). Detergents themselves have a killing effect on some microorganisms and are frequently, if not invariably, used hot. Some disinfectants may exert a detergent action.

Cosmetic and pharmaceutical creams may pose several problems, since remnants of production batches may remain in relatively inaccessible orifices and crevices in apparatus and machinery used in their preparation. Such remnants could form foci for the infection of future production batches, which in turn could influence the activity of the preservative selected for incorporation into the product. Bean (1967) recommends cleaning of apparatus and machinery, after use, with hot water and detergent, followed by an appropriate disinfectant or steam.

Davis (1972a) recommends four ways of chemically sterilizing/disinfecting equipment in the cosmetic industry.

1 Detergent, such as alkali, followed by a hypochlorite or a QAC.

2 Cleaning by a stronger concentration of detergent–disinfectant and then sterilization/disinfection by a weaker concentration.

3 Cleaning and sterilizing/disinfecting with a detergent–disinfectant (such as alkali and QAC), followed by a 'sterile rinse' with a QAC or a hypochlorite.

4 Using a single substance, such as sodium hydroxide or nitric acid, which has powerful cleaning and sterilizing properties, followed by a sterile rinse.

A publication by the British Standards Institute (Anon., 1977) is worthy of comment. This deals with recommended methods for sterilizing plant and equipment used in the dairy industry; the term 'sterilization' as used in this report means 'a process which reduces the number of bacteria in dairy plant and utensils to a level consistent with acceptable quality control and hygienic standards'. Thus, while some of the processes recommended might achieve sterilization in the normally accepted sense of the word, the present authors consider that the terms 'disinfection' and 'disinfectant' are more logical. The chemical agents described are: chlorine (see Section 9.2 in the present chapter); iodophors (Section 9.1.2); QACs (Section 6.1); amphoteric surface-active agents (Section 6.4); anionic surface-active agents (Section 6.2) with an inorganic acid, usually phosphoric acid, to give highly acid solutions for removing and preventing milkstone; sodium hydroxide; and formaldehyde (Section 7.2). The report provides useful information on the inclusion of detergents into the formulation to provide balanced products which clean, which are microbicidal and which can be employed below 60°C. At temperatures of 70°C and above, the detergents alone are able to kill most spoilage and pathogenic bacteria. Of prime importance are the compatibility of the two ingredients (in particular, the fact that activity of a microbicidal agent may be enhanced or reduced by a detergent; see Chapter 3) and the need to avoid an increase in the risk of corrosion of the plant or equipment. In the latter context, it is of interest to note that the incorporation of suitable alkaline agents reduces the risks of corrosion induced by chlorine-releasing agents.

Ultrahigh-temperature (UHT) plant in the dairy industry requires true sterilization (as opposed to disinfection see above) and for this pressurized hot water at 140–150°C is recommended in the report.

Finally, mention should be made of some studies by Muys *et al.* (1978), who investigated hydrochloric acid vapour as a sterilizing agent for heat-sensitive food containers. The aim of this work was to obtain a rapid low-temperature method in

which no toxic residues remained, as occurred with other vapour-phase chemicals, such as ethylene oxide, hydrogen peroxide, methyl bromide and propylene oxide. Such residues are unacceptable because they could contaminate food packed in the treated containers. The sporicidal activity of hydrochloric acid vapour in this investigation suggests that it is worthy of further study.

21 Disinfectants in recreational waters

The growing popularity of public and private swimming-pools has led to the inevitable problems of maintaining adequate hygienic standards, notably in relation to the possible transmission of infective microorganisms from one person to another. At the same time, control measures must ensure that the swimming-pool water has no toxic or irritant effects on the users of the pool. Various microorganisms have been associated with infections arising from hydrotherapy pools, swimming-pools and whirlpools, but the most frequently implicated organism is *Ps. aeruginosa*, the source of which is often the pool pumps (Friend & Newsom, 1986; Aspinall & Graham, 1989). For many years, chlorine disinfectants have been employed as a sanitary control measure. In 1959, the effectiveness of iodine in the disinfection of swimming-pool water was described (Black *et al.*, 1959) and since then two important papers which compare the relative effectiveness of chlorine and iodine have been published by Black and his colleagues (Black *et al.*, 1970a,b). Iodine scored over chlorine in the following ways: free chlorine and iodine were effective pool sanitizers, but chlorine is more expensive, and iodine is more stable in dilute aqueous solution. Chlorine employment involves the drawback of maintaining adequate residual concentrations when the pool is heavily used, and its eye toxicity is another factor that must be considered; in contrast, its instability can be considered advantageous in terms of keeping a pool free from organic matter and free available chlorine is active in controlling algae. Iodine is ineffective against algae, and thus cannot be recommended for the disinfection of swimming-pool water until suitable formulations can be developed which overcome this disadvantage. Another useful agent used for the disinfection of swimming-pools is the polymeric biguanide, Baquacil SB (Imperial Chemical Industries, Manchester M9 3DA). The properties of this type of compound have been described in Section 5.3.

Warren *et al.* (1981) have published a comparative assessment of swimming-pool disinfectants. Problems arising from the increasing use of whirlpools are referred to in Report (1989).

Hydrotherapy pools are the subject of a later chapter (Chapter 15).

22 Other uses of antimicrobial agents

Antimicrobial agents are used widely as disinfectants and antiseptics in the hospital and domestic enviroments, as preservatives or bactericides in sterile or non-sterile pharmaceutical or cosmetic products (Hodges & Denyer, 1996), and as preservatives in certain foodstuffs. Additionally, they are employed in certain specialized areas, such as cutting oils, fuels, paper, wood, paint, textiles and the construction industry. Some of these aspects are considered in detail in later chapters.

23 Which antimicrobial agent?

23.1 Regulatory requirements

The Federal Drug Administration (FDA) in the USA, the EU for the European community and most other countries publish information on the permitted use and concentration of preservatives. Current regulations should be consulted and complied with when manufacturing in these countries and exporting to them.

The situation from the American point of view has been reviewed by Eirmann (1984). Greenwood (1990) has provided a very comprehensive overview for preservative use over a wide range of countries.

Cosmetic preservatives allowed in the EU are described by Hill (1995), who also considers what percentage, if any, of each is permitted for use in US cosmetic formulations. In the UK, the Ministry of Agriculture, Fisheries and Food (MAFF) publishes information on food additives and E-numbers.

23.2 Which preservative?

Because of the many variables which affect the activity of antimicrobial agents, it is almost impossible from a mere scrutiny of the literature to select a preservative that will be optimal in a particular product. Legislation passed in the USA by the FDA required the manufacturers of cosmetics to declare the ingredients in their products and to state their function or purpose. This information was computerized and the data relating to declared preservatives published (Richardson, 1981). In this survey 19 584 formulae from 902 companies were included. This list of the 10 most used antimicrobial agents, with the number of times they appeared in the 19 584 submissions processed, was as follows:

Methyl *p*-hydroxybenzoate	6785
Propyl *p*-hydroxybenzoate	6174
Imidazolidinyl urea	1684
N-(3-*cis*-chlorallyl)hexamine	1001
Formaldehyde	874
Butyl *p*-hydroxybenzoate	668
2-Bromo-2-nitropropan-1,3-diol	566
Sorbic acid	393
Sodium dehydroacetate	191
Ethyl *p*-hydroxybenzoate	159

Although this is a statistical, rather than a scientific, survey, it does represent the combined expertise of a large number of organizations. Unfortunately, this list did not indicate if and where combinations of preservatives were used.

As regards combinations, an appraisal of the literature seems to suggest that a combination of one of the more water-soluble esters of *p*-hydroxybenzoic acid, probably the methyl ester, together with one of the water-soluble urea derivatives or a sulphydryl reactive compound, might be a good combination to start with. Denyer *et al.* (1985) have discussed synergy in preservative combinations.

If the product is a water-in-oil emulsion, and it is felt that the oily phase needs protection, especially from mould infestation, then a third component, one of the oil-soluble esters of *p*-hydroxybenzoic acid, e.g. the butyl ester, or an oil-soluble phenol, such as *o*-phenylphenol, might well be added. Over and above this, there remains the question-begging proviso 'providing other criteria such as compatibility, stability, toxicity and regulatory requirements are satisfied'.

23.3 New concepts

In recent years, 'natural antimicrobial agents' have increasingly been considered by food microbiologists as potential preservatives for food products. These agents may be associated with immune systems and have been examined in mammals, insects and amphibians. As pointed out by Board (1995), an agent active against prokaryotic but not mammalian cells is of obvious interest. Although Board (1995) was discussing natural antimicrobials from animals as potential food preservatives, it is clear that their possible use in other areas should also be investigated.

Likewise, natural antimicrobials from plants (Nychas, 1995) merit further consideration. It is of interest to note here that 'natural and physical preservative systems' are also being considered as an important part of the production of cosmetic and non-sterile pharmaceutical products (Leech, 1988). Such systems refer to the utilization of pH, natural antimicrobial agents or antimicrobial formulation components. Aspects have already been discussed in this chapter or will be considered in Chapter 3, which presents data about the influence of various factors on antimicrobial activity.

24 The future

New biocidal agents are unlikely to be produced in the foreseeable future, although it might be possible to modify chemically some of the existing compounds with the aim of enhancing their activity. Such a procedure has worked well with chemotherapeutic drugs.

With the emergence of 'new' pathogenic entities, such as the prions, glycopeptide-resistant enterococci and multidrug-resistant mycobacteria, as well as biocide-resistant mycobacteria, it is clear that better usage of existing biocides is necessary. This has been discussed by Russell & Russell (1995) and Russell & Chopra (1996). In brief, future policies might well be to examine combinations of biocides, or of a biocide with a permeabilizer, to re-evaluate older, perhaps dis-

carded, molecules, to consider whether physical procedures can enhance antimicrobial activity and, where relevant, to determine how natural antimicrobial systems can be better utilized.

A long-term goal should be the achievement of a better understanding of the ways in which microorganisms are inactivated and of the mechanisms whereby they circumvent the action of a biocide.

Current knowledge about these aspects will be found in the subsequent chapters that form Part I of this book.

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